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14920 P

Transformation of Adult Schwann Cells Into Macrophages.*

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As was shown previously by one of us,¹ embryonic Schwann cells in tissue culture can transform into macrophages. The following experiments prove the same faculty for the Schwann cell of adult nerve.

In a total of 574 tissue cultures, over 1,000 fragments of peripheral nerves of white rats were cultured for 2 to 9 days in fowl blood plasma with traces of chick embryo extract. Both freshly transected and predegenerated (2 to 100 days) nerves were used. Growth was excellent and no harmful effect of the heterologous medium was noticed. Schwann cells and "fibroblasts" migrate from the explanted fragment in the known manner.^{2,3,4} These two cell types are sharply distinguished by size, nuclear properties, staining reaction, etc.

During cultivation, the culture medium undergoes certain changes (Fig. 1). A protein-rich exudate spreads from the explant (E) along the mica cover slip (M), and a liquid layer (L) separates the central portion of the plasma clot (P) from its support (M). Thus 6 different zones become evident: (1)

interface mica-liquid; (2) interior of liquid; (3) interface liquid-plasma clot; (4) interior of clot; (5) outer surface of clot; (6) interface clot-mica. Each one of these zones has its peculiar physicochemical properties and ultrastructural pattern, and the behavior

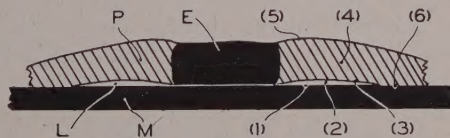


FIG. 1.

* The research reported in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. It was aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

¹ Weiss, P., *Anat. Rec.*, 1944, **88**, 205.

² Ingébrigtsen, R., *J. Exp. Med.*, 1916, **23**, 251.

³ Murray, M. R., and Stout, A. P., *Am. J. Path.*, 1940, **16**, 41; *Anat. Rec.*, 1942, **84**, 275.

⁴ Abercrombie, M., and Johnson, M. L., *J. Exp. Biol.*, 1942, **19**, 266.

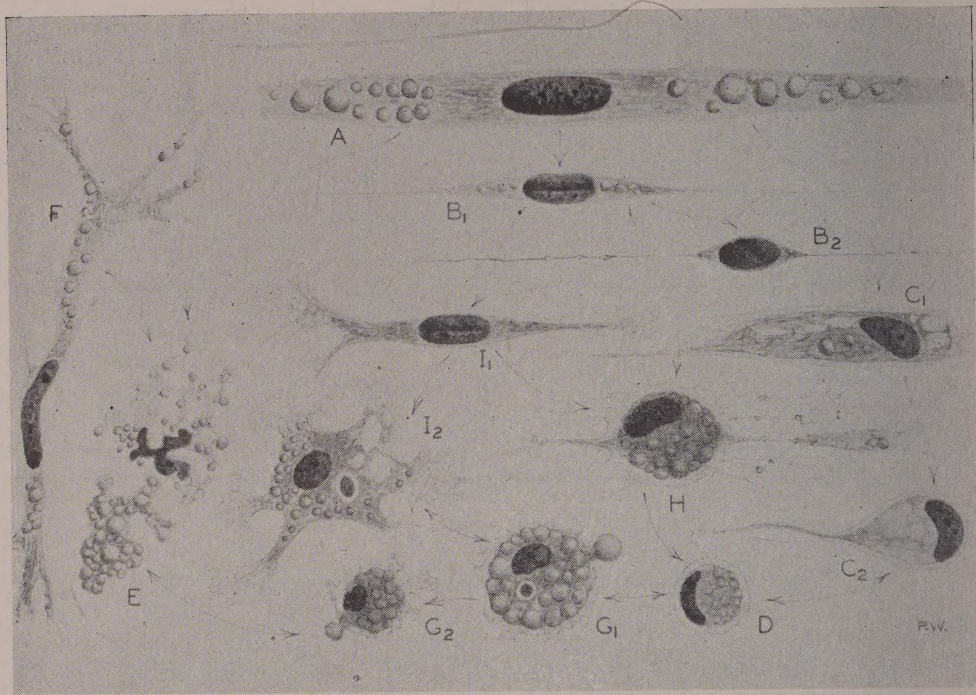


FIG. 2.

and shape of the cells entering it vary accordingly, as is more fully explained in the following summary based on observations of both living and fixed cultures.

The Schwann cells emerge from the neurilemmal tubes in tandem file, either singly or in bands (Büngner's cords). Depending on the conditions which they encounter, they may then assume any of the shapes and characters illustrated in Fig. 2. All cells are reproduced in correct proportions. Size differences are partly real (*e.g.*, B₁-B₂; G₁-G₂), partly simulated by extreme flattening (*e.g.*, I₁-I₂). All cells leave the nerve in forms A, B₁ or F. Transformations observed regularly are indicated by arrows; reciprocal arrows indicate reversible changes. Linear spindle cells (B₁, B₂) are filaments less than 1 μ wide (except near the nucleus) and up to 0.5 mm long; their ends taper to 0.2 μ and below. They prevail in layers 4 and 5. Cells in surface 1 expand by means of terminal pseudopodia and transform into macrophages (B₁→I₁→I₂) in the same man-

ner as described for embryonic Schwann cells.¹ In surfaces 3 and 5, transformation to a monocyte-like round cell occurs by retraction of the cell processes (B₁→H→D), sometimes by way of a monopolar phase (A→C₁→C₂→D). In area 6, the cells assume the highly ramified form E with polymorphic nucleus, a form also assumed by Schwann cells emerging from the nerve in the form F. All forms may round up into forms G₁ and G₂, which are highly phagocytic and in active amoeboid motion.

These transformations are accompanied by changes in staining properties, nuclear shape and size, hydration, fat content, and mode of locomotion. They were so abundant in most cultures that they could be studied in great detail in many thousands of specimens. High motility, mitotic activity and phagocytosis distinguish these transforming cells from the moribund types often seen in old tissue cultures. Mitoses were repeatedly observed in A, B₁, F, H, D and G. Phagocytosis of ink particles was intense in I₂ and G. Fat

was being actively extruded from E and G. Myelin fragments (from early nerve degeneration stages) are actively ingested by forms I and G, and their subsequent intracellular digestion has been followed under the microscope.

Transformation of Schwann cells to macrophages can also be seen inside the explanted nerve fiber tubes themselves. Removal of blood from the nerves prior to explantation (Ringer's perfusion of the donor) and Trypan Blue injections indicate that the contribution of hematogenous and preformed histiocytes to the macrophage population of our cultures was negligible. Most of the macrophages present were transformed Schwann cells, and all cultures exhibited some transformation. Fibroblasts (endoneurial cells) also turned into macrophages, but less readily; moreover, phagocytes from Schwann cells and those from fibroblasts each retain distinguishing features of their strain. The observed conversions thus belong clearly in the class of cell "modulations".^{5,6}

Conclusions. (1) The adult Schwann cell is capable of a wide range of modulation in the course of which it can assume numerous shapes and characters often associated in neuropathology with distinct cell types. (2) Schwann cells presumably are a major source of macrophages during Wallerian nerve degeneration, and their control becomes a prime consideration in nerve regeneration.⁷ (3) The active role of the Schwann cell in removing myelin by phagocytosis and digestion has been experimentally verified. (4) The filamentous shape of the spindle-type Schwann cell makes it a superb guide for axons.⁸ Its dimensions and staining properties are very close to those of axon sprouts, which suggests one source of error in past unfounded claims of "autogenous" nerve regeneration. (5) The results place increased emphasis on the great versatility of even the adult cell, within the limited potentialities of its strain, in response to the composition and microstructural organization of its environment.⁵

⁵ Weiss, P., *Principles of Development*, 1939, Henry Holt & Co., New York.

⁶ Bloom, W., *Physiol. Rev.*, 1937, **17**, 589.

⁷ Weiss, P., *J. Neurosurg.*, 1944, **1**, 400.

⁸ Weiss, P., and Taylor, A. C., *Arch. Surg.*, 1943, **47**, 419.

14921

III. Mechanism of Stomal Ulcer Is Related to Length of Afferent Duodenojejunal Loop.*

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The two preceding papers (I and II)^{1,2} in this series of experiments suggest definitely that the length of the afferent duodenojejunal loop is an item of real importance in deter-

mining whether or not stomal ulcer will follow the Schmilinsky operation or gastric

* The researches upon which this presentation is based were supported by the Augustus L. Searle Fund for Experimental Surgical Research, the Citizens' Aid Society, the Harry B. Zimmerman Fund for Experimental Surgical Research and by grants of the Graduate School of the University of Minnesota.

¹ Merendino, K. A., Varco, R. L., Litow, S. S., Kolouch, Fred, Jr., Baronofsky, Ivan, and Wangenstein, O. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, **58**, 222.

² Merendino, K. A., Lannin, B. G., Kolouch, Fred, Jr., Baronofsky, Ivan, Litow, S. S., and Wangenstein, O. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, **58**, 226.

³ Kolouch, Fred, Jr., *Surgery*, in press.

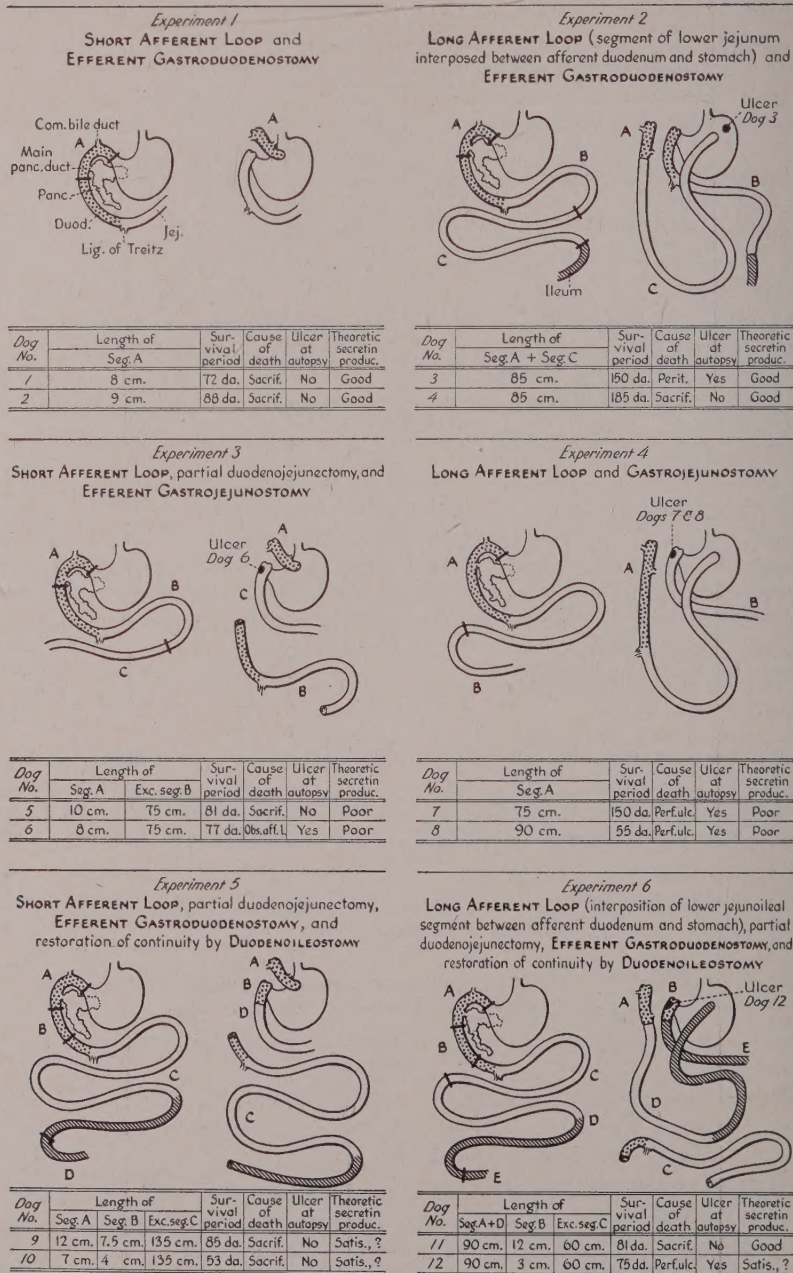


FIG. 1.

Master chart indicating the 6 types of operative procedures invoked to study the "secretin, distance and sensitivity" factors in 12 dogs. The results of the experiments also are shown.

TABLE I.
Data Relating to Experiments Carried Out to Test Importance of Secretin, Distance and Sensitivity Factors

Dog	Length of afferent loop cm	Position of efferent stoma	Theoretic secretin production	Length of post-operative life days	Cause of death	Ulcer at efferent stoma
1	short (8)	Duodenum	Good	72	Sacrificed	No
2	" (9)	"	"	88	"	"
3	long (85)	"	"	150	Died; peritonitis due to perforated gastric fundal ulcer	"
4	" (85)	"	"	185	Sacrificed	"
5	short (10)	Jejunum	Poor	81	"	"
6	" (8)	"	"	77	Died of obstructed afferent loop	Yes
7	long (75)	"	"	150	Died; perforated stomal ulcer	"
8	" (90)	"	"	55	" " " "	"
9	short (12)	Duodenum, short segment (7.5 cm)	Questionably satisfactory	85	Sacrificed	No
10	" (7)	Duodenum (4 cm)	Questionably satisfactory	53	"	"
11	long (90)	Duodenum (12 cm)	Good	81	"	"
12	" (90)	Duodenum (3 cm)	Questionably satisfactory	75	Died of perforated stomal ulcer	Yes

resection carried out on the Billroth II plan of operation. The purpose of this paper is to attempt to determine, if possible, the relative importance in the occurrence of stomal ulcer of the 3 factors suggested in the first presentation. It is believed by varying the conditions of the experiment that, the significance of each of these 3 factors may be subjected to experimental scrutiny. The 3 items to be examined with respect to their importance in the genesis of stomal ulcer are: (1) The secretin factor (2) The factor of spatial separation of alkaline and acid digestive secretions (3) The sensitivity factor, implying an increased susceptibility of the mucosa of successively lower segments of the small intestine to injury by the acid gastric secretions.

Method. Six modifications of the total intragastric duodenal drainage operation of Schmilinsky and McCann were carried out in a series of 12 dogs. The operations were devised to study the influence of both short and long afferent duodenojejunal loops on the development of stomal ulcer just beyond the efferent gastric outlet with special reference to an attempt to evaluate the significance of the 3 factors enumerated above. In other words, in addition to varying the length of the afferent loop, the site of the

efferent outlet of the stomach was varied, permitting testing of the importance of the secretin factor and the item of mucosal susceptibility to corrosion by the acid gastric secretions. These latter objectives of the study necessitated the making of some rather complicated operative procedures. By transection of the duodenum just beyond the major pancreatic duct and interposing a loop of jejunum between the proximal portion of the duodenum and the stomach, (or by excision of a portion of the duodenum and the upper jejunum in other experiments), it became possible to vary all the factors which we wished to scrutinize. In some experiments the afferent loop was long, yet the requirements of a functional secretin mechanism were met satisfactorily by placement of the entire length of the duodenojejunal segment (beyond the major pancreatic duct), at the efferent gastric outlet. By interposition of a short segment of duodenal mucosa between a high ileal segment and the gastric outlet, it was possible to note when stomal ulcer followed, whether it occurred in the short duodenal segment or in the more susceptible high ileal mucosa beyond.

Results. The results of the experiments are shown in Table I and in the graphic record of the operations. (Fig. 1). Five of

TABLE II.
Incidence of Occurrence of Efferent Stomal Ulcer as Related to Theoretic Efficiency of Secretin Mechanism.

Theoretic quality of secretin mechanism	No. of dogs	No. of dogs developing ulcer	Incidence in %	Anticipated incidence in % if correlation was perfect
Good	5	1	20	0
Questionably satisfactory	3	1	33.3	
Poor	4	3	75	100

TABLE III.
Incidence of Efferent Stomal Ulcer as Related to Spatial Separation of Source of Alkaline Duodenal Juice from the Gastrojejunostomy Stoma.

Length of afferent duodenojejunal loop	No. of dogs	No. of dogs developing ulcer	Incidence in %	Anticipated incidence in % if correlation was perfect
Short	6	1	16.2	0
Long	6	4	66.6	100

TABLE IV.
Incidence of Occurrence of Efferent Stomal Ulcer as Related to Position of Efferent Stoma.

Position of stoma	No. of dogs	No. of dogs developing ulcer	Incidence in %	Anticipated incidence in % if correlation was perfect
Duodenum	8	2	25	0
Jejunum	4	3	75	100

the 12 dogs died of ulcer; in 4 of these, perforation was present. All ulcers were stomal in character, that is, just beyond the gastric outlet on the efferent loop, save one which occurred in the fundus of the stomach (Dog 3).

In Dog No. 6, the ulcer was not perforated, death being due apparently to obstruction of the short afferent loop—an item which probably had something to do with the occurrence of the ulcer. The dogs which did not succumb to ulcer were sacrificed at intervals of from 53 to 185 days.

In Tables II, III and IV is set forth the incidence of ulcer with reference to an examination of the factors under scrutiny in this study.

Comment. It is apparent from an analysis of the data in Tables II, III and IV that, it is difficult to separate out the eventual role of any single factor. This is especially true of the secretin and distance factors. Experiments 10 and 12 constitute an excellent example of the difficulty. In dog No. 10, the afferent loop was short; in dog No. 12 it was

long. In dog No. 10, only 4 cm of duodenal mucosa remained at the efferent outlet for the gastric secretions to glide over in provoking the usual secretin effect; in dog No. 12, only 3 cm of duodenal mucosa remained at the efferent gastric outlet. Spontaneous perforation of a stomal ulcer killed dog No. 12 75 days after the operation. No ulcer was present in dog No. 10, when he was sacrificed at 53 days. In dog No. 10, however, with the short afferent loop (7 cm) containing good secretin containing duodenal mucosa, regurgitation of gastric secretions into the short afferent loop may have sufficed to augment the secretin effect of the 4 cm duodenal mucosal segment at the efferent gastric outlet. In dog No. 12, on the contrary, retrograde regurgitation of gastric secretions into the long 90 cm afferent loop could not reach the rich secretin bearing area of the duodenal segment. This same dog No. 12 provides a striking lesson in another respect. The stomal ulcer occurred in the short (3 cm) duodenal segment (Fig. 2) at the efferent gastric outlet and not in the ileal mucosa just beyond.

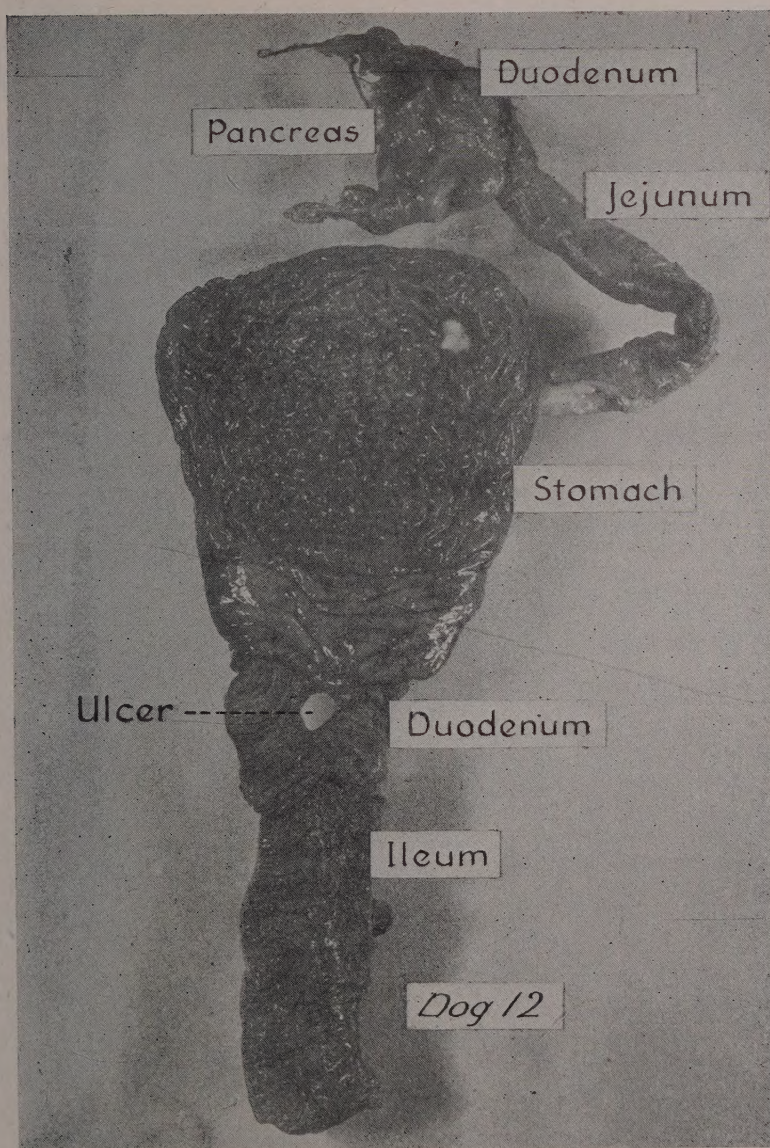


FIG. 2.

Photograph of spontaneous perforation of stomal ulcer in Dog 12. Note the position of the perforated ulcer in the small segment of duodenum interposed between stomach and ileum at the efferent gastric outlet.

In this group of experiments, stomal ulcer occurred only once in a dog with a short afferent loop (dog No. 6); in this instance, however, stenosis of the afferent inlet stoma was present, interfering with delivery of the

alkaline secretions from the duodenal loop. Moreover, in long afferent loops, in which extraneous factors might influence the motility of the segment and hence delivery of the content of the loop, it would appear that,

such long afferent loops invite stomal ulcer.

A large number of experiments in each group would undoubtedly be helpful in resolving the importance of each of the factors scrutinized in this study. In addition, the animals not dying of spontaneous perforation of a stomal ulcer should be allowed to survive longer before sacrifice.

It is not unlikely that, employment of additional modes of attack may help to separate out more definitely, the component important parts in the predisposition to stomal ulcer presented by the long afferent duodenojejunal loop. Three such methods are now being applied to the problem in this laboratory: (1) assay of the secretin potency of intestinal mucosa from varying levels of the bowel in both dog and man in dogs with pancreatic fistula. (2) Determination of the loss in titratable alkalinity, if any, of the content of the long afferent duodenojejunal loop as delivered at the afferent gastroduodenal stoma

(3) Experiments in which the sensitivity of the mucosa of various segments of the intestine is examined by allowing hydrochloric acid to drip upon isolated surfaces.

Conclusions. It is difficult to separate out with finality the role of the various factors contributing to the development of stomal ulcer attending employment of a long afferent loop in the operation of complete intragastric drainage of the content of the duodenal loop. The secretin factor can not be divorced completely from the considerations of the "distance" factor. Experiment No. 12 suggests rather definitely that, the "sensitivity" factor is not as important as the other two.

The evidence garnered in this study lends strong confirmation to the deductions arrived at in the previous two studies indicating that, a long afferent duodenojejunal loop invites stomal ulcer in any gastric operation carried out on the Billroth II plan of procedure.

14922

Effect of 2, Methyl Amino Heptane Upon Cardiac Automaticity During Cyclopropane Anesthesia in Dogs.

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(With the technical assistance of Shirley M. Wiederecht.)

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Epinephrine and certain related aromatic amines, when used during cyclopropane anesthesia, cause pronounced cardiac irregularities. Ventricular premature beats, ventricular paroxysmal tachycardia, auricular and ventricular fibrillation are the usual manifestations.^{1,2} Recently a group of aliphatic amines has been prepared which possess vasopressor activity and other pharmacologic effects of epinephrine. Studies on cardiac automaticity during cyclopropane anesthesia

using these aliphatic amines have not been reported. One of these, 2-methyl amino heptane (EA-1) is useful to overcome circulatory disturbances during spinal anesthesia.^{3,4} Inasmuch as cyclopropane is frequently used in conjunction with spinal anesthesia and may be used when this amine has been administered, it is essential to determine if it causes disturbances in cardiac rhythm and if these disturbances are similar to and as severe as those caused by epinephrine.

¹ Meek, W. J., Hathaway, H. R., Orth, O. S., *J. Pharm. and Exp. Ther.*, 1937, **61**, 240.

² Orth, O. S., Leigh, M. D., Mellish, C. H., and Stutzman, J. W., *J. Pharm. and Exp. Ther.*, 1939, **67**, 1.

³ Roman-Vega, D. A., and Adriani, J., *Current Research in Anesthesia and Analgesia*, 1944, **23**, 248.

⁴ Roman-Vega, D. A., and Adriani, J., *Southern Med. J.*, in press.

Method. Twenty-three observations were made on 5 dogs. The dogs were premedicated with morphine 1 mg/kilo and scopolamine .04 mg/kilo administered intravenously. Anesthesia was maintained at the point of intercostal paralysis with cyclopropane-oxygen mixtures using rebreathing by means of the carbon dioxide absorption technic. The amine dissolved in .5 cc of sterile water was given intravenously in one single injection over a period of 30 seconds. Studies were made on each dog before premedication, after premedication and after cyclopropane with premedication. The drug was administered from 15 to 30 minutes after the induction of cyclopropane anes-

thesia. Electrocardiographic tracings, standard leads 1, 2, 3, and lead 4 (esophageal) were taken one minute after completion of the injection at which time the pressor effect is usually established. Tracings were also made 10 minutes after the injection because the pressor action begins to disappear at this time. In 3 dogs, arterial cannulation was performed and kymographic tracings of blood pressure were correlated with the electrocardiographic studies. When the blood pressure had returned to the control level, following the use of EA-1, epinephrine .01 mg/kilo was then given and electrocardiograms taken. In dogs IV and V, carotid cannulation was not done and the order of injection reversed—

A

B

C

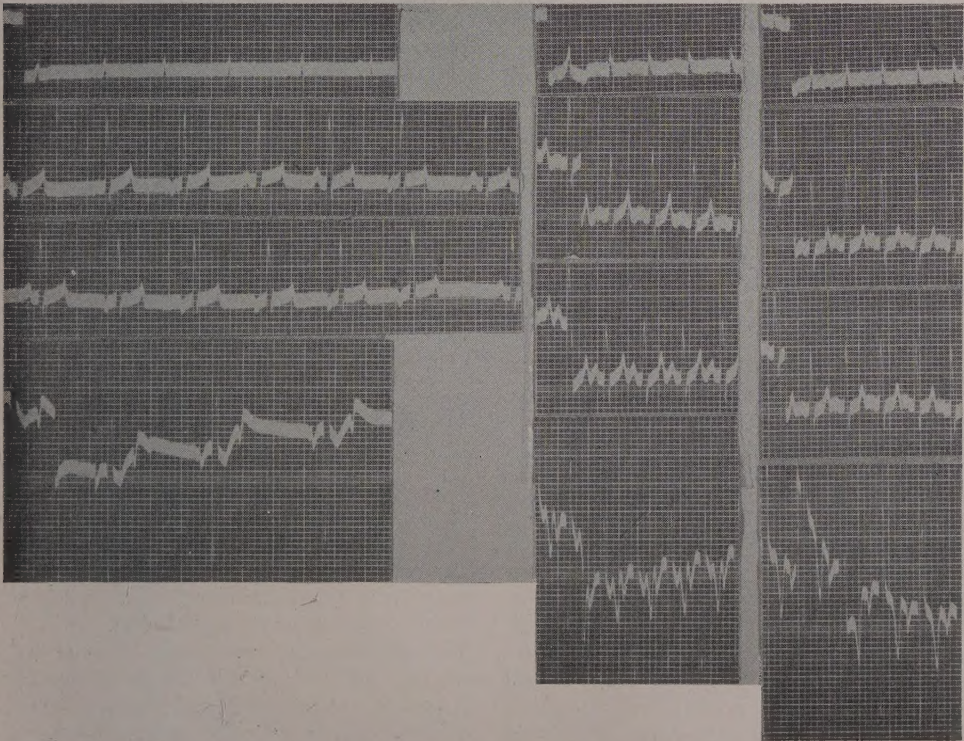


FIG. 1-A.—Dog I.—Control after cyclopropane and premedication. Sinus arrhythmia with change in P waves which may be due to shift in pacemaker or change in position of heart. (This is normal in the dog.)

FIG. 1-B.—Dog I.—One minute after EA-1. Sinus tachycardia; one to one conduction. Intraventricular conduction normal.

FIG. 1-C.—Dog I.—Ten minutes after EA-1. Rate of sinus tachycardia slightly increased. No other change.

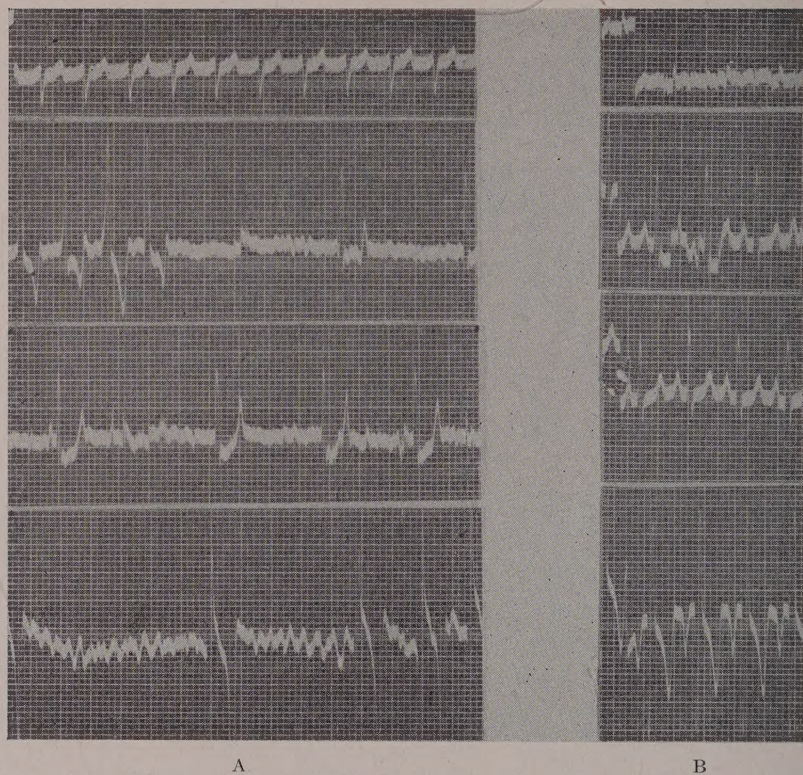


FIG. 2-A.—Dog I.—One minute after epinephrine. During the first part of the ECG there is a regular, rapid, ventricular rate possibly ventricular paroxysmal tachycardia. During this period, the auricles may be fibrillating. The tachycardia ceases abruptly in lead 2 and the mechanism for the rest of the ECG is auricular fibrillation with varying A-V conduction.

FIG. 2-B.—Dog I.—Ten minutes after epinephrine. A sinus mechanism with arrhythmia is restored.

epinephrine being given before EA-1. In dog I, the effects of excessive doses of EA-1 (2.5 mg/kilo) were also studied.

Results. In controls without premedication or anesthesia 1.0 mg/kilo of EA-1 (therapeutic dose) produced neither notable changes in conduction nor abnormal rhythms. Likewise, no notable changes followed its administration after morphine and scopolamine. In dogs I, IV and V, the administration of EA-1 during cyclopropane anesthesia with or without premedication consistently produced sinus or auricular paroxysmal tachycardia but no other disturbance of rhythm or conduction. (Fig. 1).

In dog II under anesthesia, EA-1 produced the most profound effects in the whole series

of observations. Auricular and ventricular premature beats, intraventricular block with variations in the form of QRS complexes, were observed. In dog III under anesthesia, EA-1 produced fleeting ventricular paroxysmal tachycardia in one instance. This was not produced during subsequent studies.

Epinephrine in these same 5 dogs .01 mg/kilo to .03 mg/kilo produced marked changes of conduction and rhythm *viz.*, from auricular and ventricular premature beats, ventricular paroxysmal tachycardia and auricular and ventricular fibrillation. (Fig. 2).

Cyclopropane anesthesia caused an increase in heart rate which was further increased by the administration of EA-1. No correlation was observed between ECG and

blood pressure changes. The EA-1 did not reverse the effects of epinephrine or prevent their appearance.

Comments. EA-1, in doses ranging as high as 2.5 mg/kilo, will cause disturbances of cardiac rhythm in the unanesthetized dog. Auricular paroxysmal tachycardia, auricular and ventricular premature beats are the most commonly observed disturbances. Therapeutic doses cause no such changes in the unanesthetized dog. However, in the heart sensitized by cyclopropane anesthesia, therapeutic doses can produce sufficient stimulation to cause disturbances in rhythm. These disturbances affect the auricles principally

and appear to exert little effect on the ventricular automatic tissue.

Summary. In 5 dogs premedicated with morphine and scopolamine and anesthetized with cyclopropane, 2-methyl amino heptane (EA-1) caused sinus and auricular paroxysmal tachycardia, and ventricular premature beats. Ventricular paroxysmal tachycardia and auricular and ventricular fibrillations were not observed.

The writers are indebted to Dr. James Gouax of the Heart Station, Charity Hospital for interpretation of the electrocardiograms and to Dr. Ray Parmely for assistance in anesthetizing the dogs.

14923

Inhibition of the Deciduomal Response by Preexisting Deciduomata in the Mouse*†

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Loeb^{1,2} discovered that surgical traumatization of the uterus during periods of luteal activity was followed by growth and differentiation of the endometrium into a structure which he called a deciduoma. In more recent years the observations of Krehbiel³ and Atkinson⁴ on the morphology and physiology of deciduomata have demonstrated that the reaction is the experimental equivalent of the naturally occurring maternal placenta.

* Aided by a grant, administered by Dr. Philip E. Smith, from the Rockefeller Foundation, New York.

† The progesterone (Progestin) used in this work was supplied through the courtesy of Dr. B. J. Brent of Roche-Organon, Inc., Nutley, New Jersey.

¹ Loeb, L., *Zentralbl. f. allg. Path. u. path. Anat.*, 1907, **18**, 563.

² Loeb, L., *J.A.M.A.*, 1908, **50**, 1897.

³ Krehbiel, R. H., *Physiol. Zool.*, 1937, **10**, 212.

⁴ Atkinson, W. B., *Anat. Rec.*, 1944, **88**, 271.

⁵ Selye, H., and McKeown, T., *Proc. Roy. Soc. London*, 1935, B **119**, 1.

It has been reported by Selye and McKeown⁵ that deciduoma formation cannot be elicited after placentation in the rat. Moreover, these authors found that if the pregnant horn of the unilaterally pregnant animal is removed, the remaining non-pregnant horn will quickly regain its sensitivity to traumatization. Atkinson⁶ demonstrated that this uterine desensitization to deciduoma formation is not due to changes in the estrogen-progestin balance in the pregnant mouse.

The phenomenon of uterine desensitization is difficult to analyze in the pregnant animal due to the complexity of the factors involved. However, it is possible to evaluate the role of the maternal placenta alone through the study of its experimental counterpart, the deciduoma. The present experiments were designed to determine whether the presence of deciduomata in one uterine horn would influence the responsiveness of the contralateral horn.

⁶ Atkinson, W. B., *Endocrinology*, 1944, **35**, 193.

TABLE I.
Effect of Time and Manner of Uterine Traumatization on Deciduoma Formation in Ovariectomized Mice Receiving Progesterone (1.0 mg/day).

Group	No. of animals	Day of treatment traumatized	Manner of traumatization	No. of animals developing deciduomata
1	5	3rd	unilaterally	5
		7th	contralaterally	0
2	5	3rd	bilaterally	5
3	5	7th	"	5

Experimental. Fifteen young adult mice of the Swiss albino strain were bilaterally ovariectomized. Beginning 3 to 14 days after ovariectomy the animals were given daily subcutaneous injections of 1 mg of progesterone (Progestin). Crystalline progesterone dissolved in either sesame or peanut oil in concentrations of 10 and 20 mg/cc was used. The mice were divided into 3 groups of 5 animals apiece (Table I). In the first group, one horn of the uterus was traumatized on the 3rd day of hormone treatment by placing a longitudinal suture in its lumen. Four days later (7th day of treatment) the contralateral horn was similarly traumatized. Treatment was continued and the animals were then sacrificed on the 4th day following the second uterine operation. The uteri were bilaterally traumatized on the 3rd day of hormone treatment in the animals of the second group, and on the 7th day in the 3rd group. These animals were also sacrificed on the 4th day after traumatization. All the animals were killed with illuminating gas and were autopsied immediately.

Upon examination of the uteri of the animals in the first group in which traumatization of one uterine horn was delayed until 4 days after traumatization of the contralateral horn, deciduomata were found only in the horn first stimulated. On the other hand, deciduomata were found in both horns of the mice in the second and third groups in which the uteri were traumatized bilaterally during a single operation.

Discussion. The current results demonstrate that in progesterone-treated ovariectomized mice deciduomata present in one uterine horn inhibit the development of a second set of deciduomata in the contra-

lateral horn. This failure of secondary deciduomata to develop is not due to inadequate hormone treatment or to the loss of uterine sensitivity under prolonged treatment. This is shown by the bilateral development of deciduomata in animals traumatized on either the 3rd or 7th day of hormone treatment. These observations seem to agree with the suggestion of Selye and McKeown⁵ that the placentae are responsible for the inhibition of deciduoma formation in the pregnant rat. Moreover, the present work eliminates the fetal portion of the placenta as an important factor in the desensitization of the uterus.

The current observations have a significant bearing on the problem of superfetation. Although the validity of this phenomenon has been questioned,⁷ occasional cases of true superfetation probably occur.⁸ In the normal course of events, however, the suppression of ovulation and mating during pregnancy prevents the implantation of a second set of ova in the already gravid uterus. The present work emphasizes a second and perhaps equally important factor, that of the suppression of the ability of the endometrium to differentiate into decidual tissue in the presence of pre-existing placentae.

It is well known that estrogen prevents the nidation of the egg and subsequent placentation.^{9,10} It has also been shown that deciduoma formation is dependent on a favorable estrogen-progesterone balance, the

⁷ Weichert, C. K., *Anat. Rec.*, 1942, **83**, 511.

⁸ Littleford, R. A., and Gysin, H. M., *Anat. Rec.*, 1944, **89**, 507.

⁹ Smith, M. G., *Bull. Johns Hopkins Hosp.*, 1926, **39**, 203.

¹⁰ Parkes, A. S., Dodds, E. C., and Noble, R. L., *Brit. M. J.*, 1938, **2**, 557.

reaction being inhibited by increasing amounts of estrogen.¹¹ However, the possibility that increased production of estrogen might be responsible for uterine desensitization during pregnancy seems to have been eliminated by the observation that as much as 20 times the minimal effective dose of progesterone fails to restore sensitivity in the pregnant mouse.⁶ The true nature of the mechanism of uterine desensitization remains obscure.

Summary. When traumatization of one

¹¹ Rothchild, I., Meyer, R. K., and Spielman, M. A., *Am. J. Physiol.*, 1940, **128**, 213.

horn of the uterus of progesterone-treated ovariectomized mice was delayed until 4 days after the traumatization of the contralateral horn, deciduomata developed only in the horn first stimulated. On the other hand, deciduomata developed in both horns when the uterus was traumatized bilaterally during a single operation. Since the deciduomal reaction is the experimental equivalent of the maternal placenta, these observations suggest that the maternal placenta plays an important role in the desensitization of the pregnant uterus to decidua formation. This is an important factor in the prevention of superfetation.

14924 P

Radiosensitivity of Lymphocytes and Granulocytes *In Vitro* According to the Method of Unstained Cell Counts.*

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The method of unstained cell counts¹ is used in the present investigation to determine the effect of x-rays on the survival of cells in suspensions.

Bacteriologically sterile cellular suspensions were subjected to x-rays and incubated at 37°C. Eosin in Tyrode's solution was added to the radiated and to non-radiated suspensions and the numbers of unstained cells were determined. As in previous investigations, the cells resistant to staining with eosin were presumed to be viable. The suspensions were further studied by the preparation of smears on cover slips and staining with hematoxylin and eosin and by Wright's method.

Cellular suspensions derived from the

thymus and spleen of rabbits were exposed to 1,000 roentgen units. No changes were observed in the number of unstained cells during the first 3 hours of incubation. Following this latent period, the unstained cell count decreased rapidly; after 24 hours, nearly all the cells were stainable with eosin. In control suspensions incubated for 24 hours most of the cells remained resistant to staining.

Exposure of thymic cells to 50r caused a slight but definite decrease in the number of eosin-resistant cells after 24 hours of incubation. Between 50 and 1000r, the greater the dosage, the greater was the decrease in the number of unstained cells after incubation.

Stained smears of thymic suspensions, both before and after incubation for 24 hours, had numerous lymphocytes which were normal in morphologic appearance. Degenerated, poorly stained cells were few in number in the smears of the fresh suspension and were moderate in number after incubation. In contrast, nearly all the lymphocytes in smears of radiated and incubated suspensions were

* Published with the permission of the Medical Director of the Veterans Administration who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.

¹ Schrek, R., *Am. J. Cancer*, 1936, **28**, 389; *Arch. Path.*, 1943, **35**, 857; *ibid.*, 1944, **37**, 319; *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 283; *ibid.*, 1944, **57**, 348.

definitely degenerated and only a few cells were normal in appearance. It was evident that exposure to x-rays and incubation for 24 hours caused marked degenerative changes in the thymic cells.

Suspensions from bone marrow of rabbits were radiated with 1000r and incubated. No differences were observed in the unstained cell counts of radiated and non-radiated suspensions. The granular cells of the bone marrow, unlike the lymphocytes of the thymus and spleen, were apparently resistant to radiation.

The leukocytes of normal human blood were radiated with 1000r and incubated for a period of 7 days. The number of eosin-resistant cells in the radiated suspensions decreased at a more rapid rate than those in the non-radiated suspensions. A study of smears of blood cells in suspensions indicated that the neutrophilic polymorphonuclear leukocytes were destroyed by incubation for 2 to 3 days, the lymphocytes were killed by radiation plus incubation but the eosinophilic leukocytes were resistant to both radiation and incubation.

Suspensions were prepared of the leukocytes from the blood of 12 patients with

leukemia. Unstained cell counts and smears indicated that the leukocytes of lymphocytic leukemic blood were radiosensitive in 4 of the 5 cases, but the cells of myelogenous leukemia, in all 7 cases studied, were resistant to the action of 1000r.

The effect of various factors on the radiosensitivity of lymphocytes was studied. It was observed in preliminary experiments that x-rays had no definite effect on lymphocytes when the radiation and incubation occurred under anaerobic conditions.

Summary. Suspensions of lymphocytes were derived from the thymus and spleen of rabbits and from normal and lymphocytic leukemic blood of patients. Radiation with 1000r and incubation at 37°C, caused the death of the lymphocytes after a latent period of 3 hours. Radiation with as little as 50r had a minimal cytotoxic effect on thymic lymphocytes. In contrast, granulocytes in suspensions from the spleen and bone marrow of rabbits and the leukocytes of myelogenous leukemic blood of patients were resistant to radiation with 1000r. It appeared that radiation and incubation had a delayed cytotoxic action on lymphocytes but not on granulocytes *in vitro*.

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Adaptation to Changes in Environmental Humidity at Constant Temperatures.

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Much attention has been given in recent years to the physiological reactions associated with climatic changes. Special interest was focused on the response of the body to a sudden change in one or another factor in the climatic environment (temperature, humidity, barometric pressure), rather than to its behavior in a given environment. It is assumed that these adaptation reactions may throw light on the regulations occurring in

the body during acclimatization. Bazett, Sunderman and Scott¹ studied the alteration in the blood volume in humans, and Burton, Scott, McGlone and Bazett,² the alteration in heat exchange and in the partition of heat loss, following changes in temperature condi-

¹ Bazett, H., Sunderman, F. W., and Scott, J. C., *Amer. J. Physiol.*, 1940, **129**, 69.

² Burton, A. C., Scott, J. C., McGlone, B., and Bazett, H., *Amer. J. Physiol.*, 1940, **129**, 84.

* Died on September 23, 1944.

tions. Gelineo³ and Lee,⁴ investigated the basal metabolism of rats and rabbits, respectively, following changes in environmental temperature. Silvette⁵ described the effect of changes in the barometric pressure on the urine excretion of rats.

The study reported in this paper deals with a third climatic factor: relative humidity. In this investigation we studied the effect of sudden changes in relative humidity at constant temperatures on water intake and on the urine, Cl and N excretion of rats.

Method. The experimental rats were kept in metabolic cages, two in each cage. These cages were placed in closed tin containers immersed in a water bath of a constant temperature of 28°-30°C. The containers were aerated by a constant current of air which was passed first through a bottle of sulfuric acid or a bottle of water, according to whether a low or high relative humidity was desired. The stream of air was regulated to give a temperature of 28°-30°C and relative humidities of 10%-20% and 80%-100% respectively in the cages.

The animals received a diet of the following composition: Casein 13%, rice flour 63%, butter or vitaminized margarine 8%, olive oil 2%, Marmite 10%, salt mixture 4%. Food and water were given without limit. The water was served from a glass tube inserted in bottles placed in an inverted position on top of cage; the aperture of the tube was so small that water could be removed only by licking. This arrangement made it possible to measure fairly accurately the quantity of water consumed by each pair of rats during a given period. The urine was collected in test tubes attached to the funnel of the metabolic cages. Every two days we measured the quantities of water consumed and of urine excreted and determined the Cl and N content of the urine.

The animals were kept at a given set of

conditions for 2 to 3 weeks, until there were only small variations in the amounts of urine, Cl, and N excretion. At this stage the rats were transferred from the "dry" container to the "humid" and from the "humid" to the "dry" and the tests continued. In this way no changes were affected in the conditions under which the animals were kept with the exception of the single variable humidity.

Results. The results are represented in Table I. It can be seen that:

a) when rats acclimatized to a "dry" atmosphere are transferred to a "humid" one, they react with a statistically significant increase in the excretion of urine, Cl, and N. This effect is noted during the first days after the transfer and continues for 4-8 days. The increased urine excretion is not accompanied by a larger water intake.

b) on the other hand, rats acclimatized to a "humid" environment consume about 19% more water after being transferred to the "dry" cages. The daily amounts of urine, Cl, and N excreted are diminished. This decrease, however, is very irregular and does not appear to be statistically significant. The effect, so far as it does occur, is seen only during the first days after the transfer and disappears after about one week.

The results obtained evidently represent a reaction of the organism to a sudden change in external conditions and disappear again after the body has adapted itself to its new environment. It is noteworthy, that the effect observed after transfer from a "dry" to a "humid" environment, *viz.* increase in excretion of water, Cl, and N, is similar to that following stimulation of the thyroid gland. It seemed, therefore, likely, that the sudden change in the surrounding conditions from "dry" to "humid" activated the thyroid gland. If this assumption is correct, then thyroidectomized rats should not react in a similar manner to such a change. To test this hypothesis the experiments—change from "dry" to "humid"—were repeated with thyroidectomized rats. Each experiment was carried out with two thyroidectomized rats, the normal rats of the same age and sex serving as controls; the two sets were kept in the same tin container. The results are

³ Gelineo, S., *Bull. Acad. Sci. Math. Nat. Belgrade*, B., 1940, **6**, 149; *cit.* Carpenter, T. M., *Energy Metabolism*, *Ann. Rev. Physiol.*, 1941, **2**, 144.

⁴ Lee, R. C., *J. Nutr.*, 1942, **23**, 83.

⁵ Silvette, H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 199.

TABLE I.
The Influence of Change in Humidity Conditions at Constant Temperatures.
(Each number represents the average value per rat per day for a period
of 7 days before and 7 days after the change in condition.)

	Period before the change				Period after the change			
	Water cc	Urine cc	Cl mg	N mg	Water cc	Urine cc	Cl mg	N mg
Normal rats, Dry					Humid			
No. of exper	26	26	26	18	26	26	26	18
Mean (M)	17.6	4.0	18.2	50	18.0	6.3	28.0	87
Standard deviation, σ	4.19	1.60	8.19	21.6	4.98	3.23	9.64	36.4
Standard error, ϵ	0.82	0.32	1.60	5.1	0.98	0.63	1.90	8.6
Increase in water intake:				not significant	R*	0.26	P†	0.8
" " urine excretion:				"	R	3.2	P	0.01
" " Cl				"	R	4.0	P	0.01
" " N				"	R	3.7	P	0.01
Normal rats, Humid					Dry			
No. of exper	19	19	19	13	19	19	19	13
(M)	13.7	4.0	18.7	48	16.3	3.4	15.9	39
σ	3.41	2.08	9.45	31.3	4.08	1.98	7.26	21.7
ϵ	0.78	0.48	2.17	8.7	0.94	0.45	1.66	6.0
Increase in water intake:				significant	R	2.1	P	0.05
Decrease in urine excretion:				not	R	0.9	P	0.4
" " Cl				"	R	1.0	P	0.3
" " N				"	R	1.0	P	0.3
Thyroidectomized rats, Dry					Humid			
No. of exper	4	4	4	4	4	4	4	4
(M)	15.0	4.2	18.0	55	14.5	4.1	18.0	57
σ	4.69	1.62	4.85	13.0	5.69	1.47	3.84	9.75
ϵ	2.35	0.81	2.29	6.5	2.86	0.74	1.92	4.87
Decrease in water intake:				not significant	R	0.1	P	0.9
" " urine excretion:				"	R	0.1	P	0.9
" " N				"	R	0.8	P	0.5

$$* R: \frac{M_1 - M_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$$

† P: Probability of occurring by mere chance.

shown in Table I. It appears that thyroidectomy abolishes the effect noted in normal rats following transfer from "dry" to "humid."

Discussion. The effect described by us is not the result of the prevailing humidity conditions in themselves; it is rather the reaction of animals adapted to a certain humidity to a sudden change in these conditions. It may be said, therefore, to represent a phenomenon of acclimatization. Since thyroidectomized rats did not react to a change in humidity, it would appear that the reaction appearing after transfer from a lower to a higher humidity was due to a stimulation of the thyroid gland.

Summary. (1) Rats acclimatized to a cer-

tain environmental temperature and relative humidity (10%-20% and 80%-100% respectively), were transferred to cages with a lower or higher humidity respectively, the temperature remaining constant at 28°-30°C. After transfer from "humid" to "dry" the daily output of urine and Cl and N excretion decreased during the first few days in about 50% of the experiments. On the other hand, transfer from "dry" to "humid" was followed in 80% of the experiments by a marked increase in urine output and Cl and N excretion. This effect also lasted only a few days. (2) Thyroidectomized rats did not show the reaction appearing in normal rats after transfer from "dry" to "humid" conditions. (3) It is, therefore, concluded that, with the

temperature constant, changing the environmental humidity from "dry" to "humid" activates the thyroid gland. This activation

represents an adaptive reaction of the body to the sudden change in the surrounding humidity.

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Two New Salmonella Types Belonging to Somatic Group D.

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A. Salmonella napoli. This type was represented by 10 cultures, of which 4 were isolated from cases of gastro-enteritis in United States soldiers, 3 from apparently normal soldiers and 3 from Italian civilian food-handlers. The first culture to be typed was isolated by Captain W. H. Ewing of the 15th Medical General Laboratory from a fecal specimen of an Italian civilian food-handler. The micro-organism possessed the cultural and biochemical characteristics of the *Salmonella*. Acid and gas were produced from arabinose, dulcitol, glucose, maltose, mannitol, rhamnose and xylose. Inositol, lactose, salicin and sucrose were not fermented. Sodium citrate and dextrotartrate were utilized. Gelatin was not liquefied. The organism produced hydrogen sulphide but did not form indol.

Examination of the somatic antigens of *S. napoli* showed that it belonged to group D of the Kauffmann-White classification. Alcoholized suspensions were agglutinated to the titre of *Salmonella gallinarum* antiserum and absorption of the serum with *S. napoli* removed all somatic agglutinins for the homologous strain. Somatic antigen I was found to be lacking in 6 strains, but was demonstrated in 4 by the use of an appropriate dilution of *Salmonella senftenberg* antiserum. The somatic antigens of *S. napoli* are [I], IX, XII. . .

When the flagellar antigens of *S. napoli* were examined it was found that the bacterium displayed α - β phase variation. Phase I was flocculated by *Salmonella* H antisera containing agglutinins for antigen 1. . . When tested with absorbed sera possessing the factors v, w, z₁₃ and z₂₈ respectively, it was agglutinated

only by z₁₃. In absorption tests it completely removed H agglutinins from *Salmonella uganda*, phase 1, antiserum. The antigens of phase 1 of *S. napoli* are 1, z₁₃ . . .

Phase 2 of the microorganism was agglutinated to titre by sera derived from the β phases of *Salmonella abortus-equi* (e, n, x, z₁₆, z₁₉) and *Salmonella glostrup* (e, n, z₁₅, z₁₇, z₁₉) respectively. It also was agglutinated by e, h antiserum. When tested with absorbed sera possessing factors for h, n. . . , x, z₁₅, z₁₆, z₁₇ and z₁₉ respectively, it was agglutinated by n. . . , x, and z₁₆. Absorption of *S. abortus-equi*, β phase, antiserum by *S. napoli*, phase 2, left agglutinins for the homologous strain as well as for the β phase of *S. glostrup*. However, a serum derived from the second phase of *S. napoli* was completely absorbed by *S. abortus-equi*, phase 2. The second phase of *S. napoli* behaves in very much the same way as do *Salmonella abortus-bovis* ([I], IV, XXVII, XII. . . : b—e, n, x . . .) and *Salmonella lomolinda* (IX, XII. . . : a—e, n, x . . .), both of which have deficiencies in their β phases not explained by the lack of antigen z₁₉. The 2nd phase of *S. napoli* may be expressed as e, n, x, z₁₆ . . .

The diagnostic formula of *S. napoli* is [I], IX, XII. . . : 1, z₁₃ . . . — e, n, x . . .

B. Salmonella italiana. This type was represented by two cultures. The first was sent to us by Lieutenant Colonel Robert Hebble of a General Hospital. It was isolated from an Italian civilian who had experienced repeated attacks of severe bloody diarrhea. This culture was also examined by Major Kingston S. Wilcox of the Army Medical

Salmonella pomona and *Salmonella champaign*; Two Hitherto Undescribed Types Isolated from Fowls.*†

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Fowls have been found to be one of the greatest reservoirs of *Salmonella* infection^{1,2} and it is not surprising that undescribed types occasionally should be isolated from them. In the present paper two such cultures are described.

A. *Salmonella pomona*. This type is represented by a single culture isolated in 1941 by W. R. Hinshaw from the intestine of a poult. Since the bird which yielded the culture was one of a group employed in experimental Hexamita infection no accurate estimate of the mortality caused by the bacillus is possible.

However, Dr. Hinshaw stated that the losses in the group of which this bird was a member were no higher than those usually encountered in similar studies.

The culture in question possessed the usual morphological, cultural and biochemical characteristics of the *Salmonella*. It was a motile organism which produced hydrogen sulfide but did not form indol nor liquefy gelatin. Acid and gas were produced from glucose, arabinose, maltose, trehalose, rhamnose, xylose and dulcitol. Lactose, sucrose, inositol and salicin were not fermented. Dextro-tartrate, levo-tartrate, mucate and citrate were utilized but meso-tartrate was not attacked.

When the somatic antigens of the culture were examined it reacted strongly in *S. tel-aviv*

serum but not with serums representative of the other O antigens of the Kauffmann-White schema. In absorption tests *S. pomona* removed all agglutinins from *S. tel-aviv* O serum and likewise *S. tel-aviv* removed all O agglutinins from a serum prepared from *S. pomona*. The O antigens, like those of *S. tel-aviv*, may be expressed as XXVIII.

Examination of the flagellar antigens revealed that the organism was diphasic. Phase 1 was flocculated to the titre of serum prepared from phase 1 of *S. madelia* and in absorption tests removed all H agglutinins from the serum. Phase 1 of *S. pomona* may be designated as y. Phase 2 of *S. pomona* was flocculated by serums derived from all the nonspecific phases of the Kauffmann-White classification. When tested with absorbed serums for factors 2, 3, 5, 6 and 7 it was agglutinated only by 7 serum. In absorption tests phase 2 of *S. pomona* removed all flagellar agglutinins from serum prepared from phase 2 of *S. madelia* (1, 7 . . .) and the antigens of phase 2 are 1, 7 The antigenic formula of *S. pomona* is XXVIII: y-1, 7

B. *Salmonella champaign*. *Salmonella champaign* is represented by a single culture isolated from the liver of an adult hen and forwarded to the writer by Dr. Robert Graham. No other losses in the flock of which the hen was a member were attributed to this organism. *S. champaign* differed in its biochemical characteristics from *S. pomona* only in that it produced acid and gas from inositol. Serological examination revealed that the O antigens of *S. champaign* were unlike those of any previously described *Salmonella* type. It was not agglutinated by O serums representative of the known types. Likewise, an O serum prepared from *S. champaign* failed to agglutinate the various O antigens of the genus at a dilution of 1 to 50 although the

* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

† The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Kentucky Agricultural Experiment Station.

¹ Edwards, P. R., *Proc. 7th World's Poultry Congr.*, 1939, 272.

² Edwards, P. R., and Bruner, D. W., *J. Inf. Dis.*, 1943, 72, 58.

each having differential input, were employed synchronously. The EMF of the glass electrode against either wick electrode was correlated with a pH by standardization in buffers of known pH, and the EMF of the noble-metal electrode against either wick electrode was correlated with the redox potential of systems of known composition of oxidant, reductant and pH.

We wish to confine our present note to a few of the results obtained to date.

1. When the effect of ether used for operation has passed off in cats curarized with dihydro- β -erythroidinehydrobromide* by continuous infusion, and under constant artificial

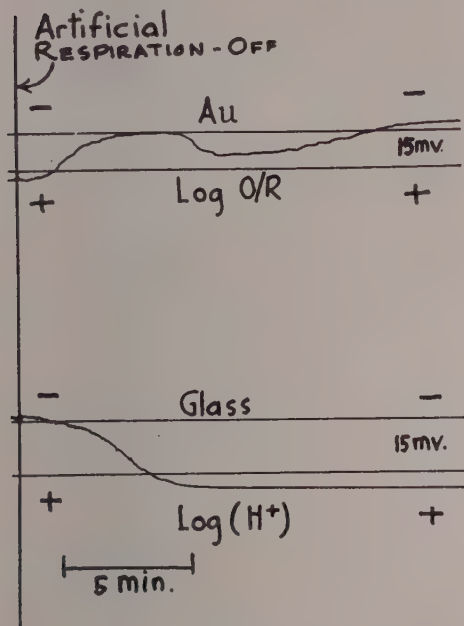


FIG. 2.

Cortex of cat under dihydro-beta-erythroidine hydrobromide. Crystographic records of amplified voltages from (top) noble-metal and (bottom) glass electrodes compared with nonpolarizable silver chloride salt bridge electrodes, showing effect of cessation of artificial respiration. Scale approximately linear throughout central 15 millivolts indicated by lines on both records. Note principal deflections are in opposite directions.

respiration, the EMF of glass and noble-metal were practically constant.

2. The DC voltages between wick electrodes under the conditions of these experiments were less than one millivolt, which was one-half the least count of the instruments.

3. The EMF of noble-metal electrodes, platinum, gold, tantalum or carbon, were not significantly different and were most frequently between .14 and .15 volts positive to the theoretical hydrogen electrode referred to which the Ag-AgCl wick electrode is .27 volts positive.

4. Alteration of composition of respired gas from 100% to 4% O₂ in N₂, although it alters the O₂ tension of cortex by a factor of 4, did not affect the EMF of either glass or noble-metal.

5. Intravenous injection of glucose was similarly without effect.

6. Injection of epinephrine decreased the EMF of both glass and noble-metal, whereas respiration of 5% CO₂ in O₂ increased both.

7. Cessation of artificial respiration produced an increase in the EMF of the glass accompanied by a decrease in that of the noble-metal. When air was readministered at the end of three minutes, both electrodes over-shot their original EMF's and returned to them slowly. (See Fig. 2.)

The equivalence of noble-metals and that they are noble incline one to believe that *in vivo* as well as *in vitro*, the observed EMF measures a redox potential. Its value is in the range of the hemochromogens. Since, in computing redox potentials, the log of the ratio of oxidant to reductant and the log of the concentration of hydrogen ion enter with the same sign, changes of both noble-metal and glass in the same direction may only indicate that the system communicating with noble-metals includes hydrogen ion, and one cannot conclude that the change involves any other significant alteration in the ratio of oxidant to reductant. However, the large changes seen upon cessation of respiration are opposite in sign, implying a great change in this ratio. For this reason, alterations of EMF of noble-metal electrodes may indicate chemical alterations which have escaped other methods of synchronous detection.

* The authors wish to thank Merck and Company, Inc., for placing dihydro- β -erythroidine at their disposal.

Relation of Adrenal Gland and Hypophysis to Blood Sugar Levels Following Administration of Alloxan.*

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Following the administration of alloxan and preceding the establishment of diabetes, the blood sugar of alloxan-treated animals showed an immediate rise which was followed by a severe hypoglycemia, the latter resulting from release of insulin by damaged islets of Langerhans.¹⁻³ Following the hypoglycemic phase a final hyperglycemia ensued. If the adrenal glands were extirpated preceding the administration of alloxan, an initial hyperglycemia did not appear, although the test animals became diabetic.³ Destruction of the adrenal medulla of rabbits by injection of formalin produced the same result as adrenalectomy.³ It was concluded that the adrenal medulla is concerned in the initial hyperglycemia.³

The present investigation was undertaken to determine the role of the adrenal cortex (and medulla) and hypophysis in governing the reaction to alloxan. Alloxan was injected either intravenously (100 mg/kg) or subcutaneously (200 mg/kg) into young adult rats which had been fasted for 12 hours. Blood sugar determinations were made at intervals after injection, as indicated below, under ether anesthesia.

Fifteen normal rats were injected with alloxan (10 IV and 5 SC). Tables I and II present blood sugar levels of these animals following alloxan administration.

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¹ Jacobs, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 407.

² Ridout, J. H., Ham, A. W., and Wrenshall, G. A., *Science*, 1944, **100**, 57.

³ Goldner, M. G., and Gomori, G., *Endocrinology*, 1944, **35**, 241.

TABLE I
Blood Sugar Levels in mg % Following Injection
of 100 mg/kg of Alloxan Intravenously into
Normal Rats.

Fasting	Hours					
	1	2	3	4	5	6
105		124		192		100
133		208			109	844
134	154	250			250	244
129		250	161			149
125				263		
138				261		
136	208	148		112	132	
102			76		66	72
128		105			71	323
140				130		167

TABLE II
Blood Sugar Levels in mg % Following Injection
of 200 mg/kg of Alloxan Subcutaneously
Into Normal Rats.

Fasting	Hours		
	4	23	
125	98	72	384
102	122	70	500
109	113	46	400
104	89	43	286
113	118	100	234

A definite initial hyperglycemia was demonstrated within 4 hours after intravenous injection of alloxan in 7 of 10 intact rats. In those injected subcutaneously the hypoglycemic phase appeared at approximately 23 hr, and the initial hyperglycemic phase, if it occurred, sometime between 4 and 23 hr after injection.

Fourteen rats were adrenalectomized 24 hr or more preceding the administration of alloxan (9 IV and 5 SC); 3 animals were operated upon immediately preceding IV injection. Three rats received alloxan IV 4 weeks following adrenal enucleation. By this procedure all of the adrenal glands except their connective tissue capsules are removed.^{4,5} From

⁴ Evans, G., *Am J. Physiol.*, 1936, **114**, 297.

TABLE III.

Blood Sugar Levels in mg% of Adrenalectomized Rats Following Injection of 100 mg/kg of Alloxan Intravenously.

Adrenalectomy performed 24 hr preceding alloxan injection				
Maintenance	Fasting	2 hr	4 hr	6 hr
Salt	92	59	45 hgc	
"	64	40 hgc*		
"	66	28 "		
"	67	41 "		
"	72	35 "		
Cortical extr	105		48 "	
"	108	44 "		
Adrenalectomy performed immediately preceding administration of alloxan				
	125	89	73	44 hgc
Animals possessing only the adrenal cortex (following adrenal enucleation)				
	118	161	161	208
	120	118	128	133
Animals from which regenerated adrenal cortical tissue was removed immediately preceding administration of alloxan				
	161		98	37 hgc
	133		85	35 "

* hgc = hypoglycemic convulsions.

the latter, functional cortices regenerate—the glands possess no functional medulla. Two animals received alloxan IV immediately following extirpation of regenerated adrenal cortices (previously enucleated glands). Regenerated adrenal cortices were serially sectioned. Of 12 such glands a single island of medullary tissue was found in one. Table III records the blood sugar levels in this series of animals.

Hypoglycemic convulsions appeared within 5 hours in fasting adrenalectomized animals injected with 200 mg/kg of alloxan subcutaneously. Compare with blood sugar levels in intact animals alloxanized with this dose (Table II).

Eight rats were alloxanized 72 hr after hypophysectomy by intravenous injection; 3 additional rats were injected IV following a sham hypophysectomy, and 2 rats were hypophysectomized immediately preceding the IV administration of alloxan. Table IV records the blood sugar levels in this series of animals.

The results indicate that the adrenal cortex rather than the medulla was concerned in the initial hyper- and hypoglycemic reactions to

alloxan. In these experiments all of 15 intact animals survived the injection of alloxan without the development of hypoglycemic convulsions. All of 14 adrenalectomized and 9 of 10 hypophysectomized rats similarly treated developed hypoglycemic convulsions within 6 hr after injection. Three of these animals were adrenalectomized and one hypophysectomized immediately preceding the injection of alloxan, indicating that the role of these glands is active, and that the rapid development of hypoglycemic convulsions in alloxanized-adrenalectomized or hypophysectomized animals is not merely secondary to an initially lower blood sugar at the moment of injection

TABLE IV.

Blood Sugar Levels in mg % of Hypophysectomized Rats Following Injection of 100 mg/kg of Alloxan Intravenously.

Hypophysectomy—72 hr preceding injection							
Pre-op	Post-op	1	2	3	4	5	6
	122				78		hgc
	80				66		"
140	125	100	83	42	hgc		
128	172	184	181				59 hgc
133	113		35 hgc*				
128	114		76 "				
139	95		32 "				
133	125		100	42 hgc			
Hypophysectomy—immediately preceding injection							
Convulsions in 8 hr.							
One animal survived injection.							
Sham hypophysectomy—72 hr preceding injection							
	125				267		
	138				264		
	140				130		

* hgc = hypoglycemic convulsions.

TABLE V.

Summary of Results Following Intravenous Administration of Alloxan (100 mg/kg)

No. of animals	Previous treatment	No. with initial hyperglycemia	No. with hypoglycemic convulsions
10	none	7	0
9	adrenalectomy	0	9
3	adrenal enucleation		
	cortical regeneration	1*	0
2	extirpation of regenerated adrenal cortex	0	2
10	hypophysectomy	0	9
3	sham hypophysectomy	2	0
Results following subcutaneous administration of alloxan 200 mg/kg			
5	none	?	0
5	adrenalectomy	?	5

* 2 animals tested.

⁵ Ingle, D. J., and Griffith, J. Q., *The Rat in Laboratory Investigation*, p. 389, 1942. J. B. Lip-pincott Co., Philadelphia, Montreal, London.

of alloxan. In 3 animals possessing only the adrenal cortex hypoglycemic convulsions did not occur following the injection of alloxan; one of the 2 animals tested showed an initial hyperglycemia. That extra-adrenal chromaffine tissue was not responsible for this protection against the hypoglycemia of convulsive level is demonstrated by the fact that hypoglycemic convulsions occurred in 2 alloxanized animals from which adrenal cortices had been removed. It would seem that the hypophyseal-corticoadrenal mechanism antagonizing the action of insulin is concerned in the initial reaction to the injection of alloxan.

Summary. An initial hyperglycemia did not appear in either adrenalectomized or hypophysectomized rats injected with alloxan. With the doses of alloxan used, hypoglycemic convulsions did not appear in any of 15 intact animals whereas convulsions occurred within 6 hr in all of 14 adrenalectomized and 9 of 10 hypophysectomized animals. In the absence of the adrenal medulla the reaction to alloxan was the same as in intact animals; removal of the adrenal cortex (regenerated cortex following adrenal enucleation) resulted in the same reaction as total adrenalectomy.

14930

Effect of Mapharsen on Bromsulphalein Retention in Normal Dogs.*

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Various arsenicals have been used as experimental agents to produce and study hepatic damage. Thus, liver injury produced by arsphenamine has been studied in relation to the protein, carbohydrate and fat content of the diet.^{1,2} More recently methionine has been reported to counteract the increased icterus index produced by Mapharsen in *protein depleted dogs*.³ Recently we studied the effect of oxophenarsine hydrochloride (Mapharsen) on the liver function of *normal dogs* and noted (1) that the bromsulphalein retention produced by a single injection of Mapharsen varied considerably and (2) that the dogs rapidly acquired a tolerance to Mapharsen, as judged by the rapid return of the liver func-

tions to normal on subsequent injections.

Methods. The dogs used in the experiments were healthy adult animals receiving a stock diet of Purina chow. After control bromsulphalein determinations were made on the animals each dog was injected intravenously with 0.06 g of Mapharsen in 10 cc of distilled water. Bromsulphalein determinations were then made 6 hr and 24 hr after the Mapharsen injection. In some of the dogs the Mapharsen injections were repeated at weekly intervals. The 5 mg/kg dose of bromsulphalein was used to estimate the degree of hepatic dysfunction, and the reported readings were read directly against the 2 mg standards. Therefore, by using the 2 mg standard the retention values may range above 100%. The control dye retentions were between 5 and 12%, so that any retention above 15% was considered as evidence of decreased liver function.⁴

Results. Of 17 dogs injected with 60 mg of Mapharsen, 59% showed a retention of bromsulphalein 6 hr later. A bromsulphalein test 24 hr after the mapharsen injection showed

* Aided by a grant from the Fluid Research Fund of Yale University School of Medicine. The authors also wish to thank Dr. Oliver Kamm of Parke, Davis and Company for the Mapharsen used in the experiments.

¹ Schiffrin, A., *Wircnows Arch. path. Anat.*, 1932, **287**, 175.

² Messinger, W. J., and Hawkins, W. B., *Am. J. Med. Sc.*, 1940, **199**, 216.

³ Goodell, J. P. B., Hanson, P. D., and Hawkins, W. B., *J. Exp. Med.*, 1944, **79**, 625.

⁴ Drill, V. A., and Ivy, A. C., *J. Clin. Invest.*, 1944, **23**, 209.

TABLE I.
Effect of a Single Injection of 60 mg of Mapharsen on Bromsulphalein Retention
in Normal Dogs.

In Normal Dogs					
Dog	Weight kg	Mapharsen mg/kg	% Bromsulphalein Retention		
			Control	After Mapharsen 6 hr	24 hr
Normal hepatic function					
1	22.2	2.7	12	8	10
2	18.6	3.2	10	12	10
3	13.1	4.6	10	10	5
4	10.5	5.7	8	12	8
5	9.8	6.1	8	8	8
6	9.1	6.6	5	8	10
7	9.1	6.6	5	10	8
Hepatic damage					
8	17.7	3.4	8	30	30
9	16.1	3.7	10	35	22
10	13.9	4.3	10	45	15
11	10.9	5.5	12	45	30
12	10.8	5.6	8	150	125
13	9.5	6.3	12	60	30
14	9.5	6.3	10	60	8
15	9.3	6.5	8	60	15
16	7.7	7.8	10	25	12
17	6.6	9.1	10	80	80

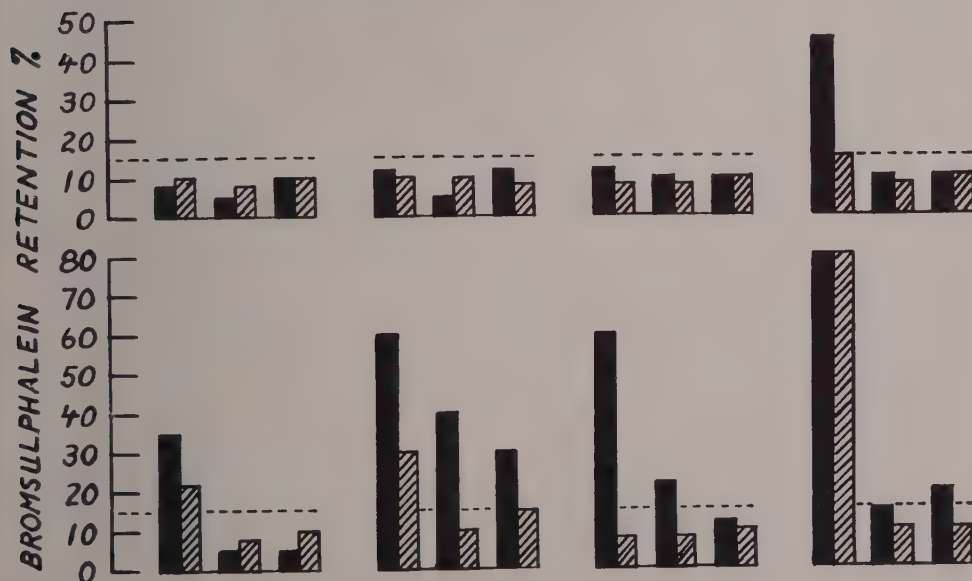


FIG. 1.

Effect of 3 injections of 60 mg of mapharsen given at weekly intervals on bromsulphalein retention of 8 dogs. Broken horizontal line indicates upper limit of normal of dye retention. Solid bar and hatched bar indicate dye retention tests 6 hours and 24 hours, respectively, after Mapharsen injection.

that the abnormal dye retention had decreased or had already returned to normal (Table I). When the dose of Mapharsen was calculated on a body weight basis no correlation between

dosage and functional impairment was observed, over the range studied (Table I).

The effect of 60 mg of Mapharsen, given once a week for 3 weeks, was studied in 8 of the

above dogs (Fig. 1). Three of the dogs did not show an increased bromsulphalein retention after the first injection of Mapharsen and subsequent injections also failed to produce demonstrable change. The remaining 5 dogs showed an increased retention of bromsulphalein following the first injection of Mapharsen. However, after the 2 subsequent injections, tolerance had increased in these dogs so that the bromsulphalein retention either approached or returned completely to normal (Fig. 1).

Discussion. Normal dogs are said to tolerate 6 to 8 mg of Mapharsen per kilo without developing liver injury, as judged by the icterus index.³ Using the bromsulphalein test impairment was detected with doses of Mapharsen as low as 3.4 mg/kg. Thus the bromsulphalein test may be more sensitive than the icterus index in detecting functional hepatic damage in dogs produced by Mapharsen. As can be seen from Table I there is also a marked variation in the liver function response following the injection of Mapharsen, so that in a smaller series of dogs one may easily obtain either a high or low percentage of the dogs showing hepatic damage. When Mapharsen was injected at weekly intervals,

dye retention which occurred following the first injection was less following each additional injection. Therefore, one cannot test a given drug against Mapharsen toxicity by injecting Mapharsen one week, and Mapharsen with the substance to be tested the following week. Rats, rabbits and dogs have been reported to acquire a tolerance to certain organic arsenicals,⁵ and the present study shows a similar tolerance with respect to the liver function of dogs.

Summary. Fifty-nine per cent of 17 normal dogs developed an abnormal liver function after a single injection of 60 mg of Mapharsen. The dye retention occurred irrespective of dosage over a range of 2.7 to 9.1 mg/kg of body weight. Dogs showing hepatic dysfunction from an initial injection of Mapharsen, subsequently showed less dye retention or a complete return to normal on 2 subsequent injections. Dogs having a normal bromsulphalein retention after one injection of Mapharsen also showed a normal dye retention following two subsequent injections.

⁵ Kuhs, M. L., Longley, B. J., and Tatum, A. L., *J. Pharm. and Exp. Therap.*, 1939, **66**, 312.

14931 P

The Prolonging Action of Penicillin by Para-Aminohippuric Acid.*

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(Introduced by B. Kramer.)

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Jewish Hospital of Brooklyn.*

The successful treatment of certain infectious diseases with penicillin, notably those in which the offending organism is resistant to the drug, certainly is compromised by the wasteful manner in which the human body disposes of penicillin. Beyer, Flippin, Verwey, and Woodward¹ have proposed para-aminohippuric acid as an agent which aids the economy whereby penicillin is utilized by the body. They report that this substance competes with penicillin for the same tubular excretory mechanism and their simultaneous intravenous administration results in augmented blood penicillin levels. In view of the

fundamental importance of this observation, it was considered worthy of clinical application in cases of subacute bacterial endocarditis particularly in those patients who were refractory to our standard dosages of penicillin.² Limited supplies of para-aminohippuric acid¹ did not permit extended studies to be made, however, and consequently only preliminary observations are being reported.

Procedure. The subjects received penicil-

* Aided by grants from friends of the Hospital and the Dazian Foundation for Medical Research.

¹ Beyer, K. H., Flippin, H., Verwey, W. F., and Woodward, R., *J.A.M.A.*, 1944, **126**, 1007.

TABLE I.
The Enhancement of Penicillin Blood Levels by the Simultaneous Administration of Para-Aminohippuric Acid.

Case No.	Penicillin Oxford units		P. A. H.		Blood levels	
	Daily dose (million)	Blood levels	Daily dose (g)	Hour of sample	Penicillin Oxf. units	P.A.H. mg %
1 A.S.	0.5	0.375	60.0		1.0	11.44
2 F.L.	0.5	0.5	100.0*		2.0	18.79
				24	3.0	18.79
				6	1.5	19.98
				24	0.43	10.04
3 M.L.	1.0	0.6	100.0*		0.43	21.60
				6	3.0	21.60
				24	4.8	11.88
4 S.Z.	2.0	2.4	60.0			

* 80 g—venoclysis + 20 g—priming.

lin[†] dissolved in a liter of solution by continuous intravenous drip, daily. The vehicle, during control periods, consisted of isotonic saline or Ringer's solution. During the experimental period the diluent consisted of sodium para-aminohippurate in 4-8% concentrations in sterile distilled water. In some experiments "priming doses" of sodium para-aminohippurate were also given twice daily. These consisted of 50 ml of a 20% solution of sodium para-aminohippurate which was injected intravenously. There were, then, 4 phases to the test: an initial control period when only penicillin was administered by continuous venoclysis, a second period when penicillin dissolved with 4-8% sodium para-aminohippurate in water was given, a third phase when priming doses of sodium para-aminohippurate were administered in addition to the maintenance dose of penicillin in sodium para-aminohippurate (second phase), and the fourth period which was similar to the first. Thus there were 2 control periods at the beginning and end of the experiment when only penicillin was administered.

The dosage schedule for penicillin varied from 500,000 to 2,000,000 Oxford units daily. The total daily dosage of sodium para-aminohippurate varied from 40 to 80 g when admin-

² Loewe, L., Rosenblatt, P., Greene, H. J., and Russell, M., *J.A.M.A.*, 1944, **124**, 144; Loewe, L., *Bull. N. Y. Acad. Med.*, Feb., 1945; Loewe, L., *J. Canad. Med. Assn.*, 1945, **52**, 1.

[†] We are indebted to Drs. Karl H. Beyer and John Henderson of Sharp and Dohme, Inc., for the supplies of para-aminohippuric acid used in this study.

[‡] We are indebted to Mr. John L. Smith of the Chas. Pfizer Co. for our supplies of penicillin.

istered without "priming," plus an additional 20 g when priming dosages were utilized. The maximum amount of para-aminohippuric acid given daily was 100 g, and the patients received this drug continuously for periods up to 8 days. In all instances penicillin assays on serum were performed at varying intervals according to the method of Rosenblatt, Altire-Werber, Kashdan, and Loewe.³ Para-aminohippuric acid levels were determined by the conventional method described by Goldring and Chassis.⁴

Results. In most instances, it was not possible to achieve high para-aminohippuric acid levels by this method, and consequently no enhancement of the blood penicillin level was apparent. However, in some 50 simultaneous para-aminohippuric acid and penicillin assays there were eight instances in which the blood para-aminohippuric acid level exceeded 10 mg %. It was found (Table I) that at these times the simultaneous penicillin level usually was 3 to 6 times the control figure. In but 2 instances were the penicillin levels below the control.

Discussion. With the technique used, it was difficult to achieve high blood levels of para-aminohippuric acid. Nevertheless, there was usually definite enhancement of the serum penicillin levels when the blood para-aminohippuric acid equalled or exceeded 10 mg %.

The enhancement of serum penicillin levels is obviously dependent upon the maintenance of high blood concentrations of para-aminohip-

³ Rosenblatt, P., Altire-Werber, E., Kashdan, F., and Loewe, L., *J. Bact.*, 1944, **48**, 599.

⁴ Goldring, W., and Chassis, H., *Hypertension and Hypertensive Disease*, Commonwealth Fund, New York, 1944, pp. 203-4.

puric acid. In order to obtain and maintain levels between 30 and 45 mg %, it is necessary to administer the drug at a rate of 150 mg/kg/hr.¹ For an average individual weighing 150 lb (68 kg), this necessitates a daily dosage of 245 g per 24 hr period. Since the daily dosage in our experiments varied between 60 and 100 g of sodium para-aminohippurate, it is not surprising that augmentation of penicillin serum levels was not consistently maintained. On the other hand, such exaggerated serum penicillin levels which were obtained take on added significance when found in conjunction with heightened para-aminohippuric acid levels.

When priming doses of sodium para-aminohippurate were administered, the same smooth muscle effects noted by Beyer and his co-workers¹ ensued. There was a feeling of restlessness and warmth. The face and neck became flushed and there was a sense of cardiac palpitation. The patients noted a burning sensation at the injection site. Several moments later there was a desire for urination and defecation. If the injection was given immediately prior to alimentation there was complete loss of appetite. These symptoms

were all mild in character, evanescent, and could be prevented by slowing the rate of injection of sodium para-aminohippurate.

There were no untoward effects noted during the simultaneous administration of sodium para-aminohippurate and penicillin by continuous venoclysis except for an immediate, occasional, slight sense of warmth along the course of the veins which disappeared in a short time. This was more likely to occur with higher concentrations of sodium para-aminohippurate. No cumulative toxic effects were observed after continuous administration of sodium para-aminohippurate for periods up to eight days.

Conclusions. 1. When the plasma para-aminohippuric acid concentration was maintained at or above 10 mg/100 ml a 3 to 6 fold elevation in penicillin plasma concentration was obtained except in two instances. 2. When the para-aminohippuric acid plasma levels were allowed to fall below 10 mg/100 ml there was no effect of that compound on the plasma concentration of penicillin. 3. The para-aminohippuric acid was non-toxic in the dosage given even when administered over a period of 8 days.

14932

Metabolism of Vitamin D in the Chick.

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In a previous publication dealing with the hypercalcemic effects of activated sterols in the chick¹ some attention was given to the metabolism of vitamin D in this species. It was found that there was excreted in the feces a larger proportion (49%) of an oral dosage of vitamin D₂ than there was of vitamin D₃ (36%). However, in the carcasses of chicks killed 48 hr after the administration of the vitamins, a larger proportion of the vitamin D₃ was found (27% as against 12%). The general purpose of other papers of this series^{2,3}

has been to obtain information which would serve to explain the remarkably low sensitivity of the chick to vitamin D₂,⁴ a characteristic which seems to be shared by turkey poults.⁵

It has been suggested¹ that there appear to be 3 possible causes for the low activity of vitamin D₂ in the chick. One of these, namely, differences in the extent of absorption from the digestive tract, has been dealt with in other papers,^{2,3} and it has been shown that such differences can account for no more than half

¹ McClesney, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 147.

² McClesney, E. W., *J. Nutrition*, 1943, **26**, 81.

³ McClesney, E. W., *ibid.*, 1943, **26**, 487.

⁴ Hess, A. F., and Supplee, G. C., *Proc. Soc. Exp. Biol. and Med.*, 1930, **27**, 609.

⁵ Willgeroth, G. B., Halpin, J. L., Halloran, H. R., and Fritz, J. C., *J. Assoc. Off. Agr. Chem.*, 1944, **27**, 289.

of the observed difference in activity. The purpose of the present work is to study the second suggested cause; namely, possible differences in the rates at which the 2 vitamins are inactivated in the tissues.

The metabolism of vitamin D in animal species has not been extensively studied. Knudsen, Remp, and Barlow⁶ have studied the metabolism of several forms of vitamin D in the albino rat. They found that the inactivation of the vitamin in the tissues proceeds very rapidly. The metabolism of prophylactic dosages of vitamin D₂ in the chick has been studied by McChesney and Giacomino.⁷

Methods. The chicks used as experimental subjects were raised as previously described.¹ When they were 32 days of age and averaged about 250 g in body weight, the actual experiment was begun. The chicks were divided into 14 groups of 4 birds each such that each group weighed between 980 and 1020 g. Half of these groups were designated for the study of vitamin D₂ metabolism, and the remainder for vitamin D₃.

The vitamin dosages were now administered orally according to the following plan: Each of the vitamin D₂ birds received, per kg body weight, 2.5 cc of a corn oil solution containing 50 mg of calciferol per cc; each of the vitamin D₃ birds received, per kg, a similar volume of a corn oil solution containing 3 mg of crystalline vitamin D₃ per cc. These doses were chosen on the basis of previous work¹ which indicated that they would produce the same degree of hypercalcemia (serum calcium elevated to 12 mg % three days after administration). On the first, second, third, fourth, fifth, seventh, and tenth days after the administration of the vitamins, a group of 4 chicks from each main group was taken for sacrifice. The birds were killed by decapitation and were immediately transferred to 2 separate flasks, each containing one liter of boiling 20% alcoholic KOH. The remainder of the procedure for the preparation of extracts for bioassay has already been described in detail.¹

Feces were collected from the vitamin D₂

and D₃ groups for the first 24 hr after the administration of the vitamins D. The excreta were thoroughly dried and then prepared for bioassay by the methods which have been described. These methods, and those used for the animals, were used by Knudsen, Remp, and Barlow in their work and were found by them⁸ to give quantitative recoveries. For this reason the present work does not include recovery experiments.

When the results of the assays* became available, it was evident that studies of the vitamin content of chicks killed at intervals less than 24 hr after the vitamin administration would be of interest. For this purpose other chicks were raised as before, but the procedure was changed slightly as follows: When the chicks were killed the entire digestive tracts and their contents were dissected out, then the remainders of the carcasses were treated as previously described. In all cases care was taken that practically all of the blood should be included in the digest.

The results have been calculated in terms of the percentage (recovered) of the dosage given, and the data are given in Fig. 1. Since the values for the amounts found in the feces cannot be represented on the graph, they are given below.

Vitamin D₂ (2 separate experiments were run): dosage given, 34,000,000 units; recovered, 17,900,000 units, or 52.6%; dosage given 12,000,000 units; recovered, 6,400,000 units, or 53.3%.

Vitamin D₃: dosage given, 2,175,000 units; recovered, 980,000 units, or 45%.

Discussion. The percentage of vitamin D₃ found in the feces in these experiments is somewhat higher than previously reported.^{1,9} The amounts of vitamin D₂ found in the feces are in good agreement, and also slightly higher than previously reported. In the chicks which were killed 8 hr after the administration of the vitamin D₃, 57% of the dosage given was recovered. This value, together with the amount found in the feces, accounts for all of

⁶ Knudsen, A., Remp, D. G., and Barlow, O. W.,

Proc. Am. Chem. Soc., 1941, Sept., 20B.

⁷ McChesney, E. W., and Giacomino, N. J., *J. Nutrition*, 1945, **29**, 229.

⁸ Personal communication from Dr. A. Knudsen.

* The author is indebted to Mr. Donald Seplin and Mrs. Charles Miller for the bioassays.

⁹ Klein, D., and Russell, W. C., *J. Biol. Chem.*, 1931, **93**, 693.

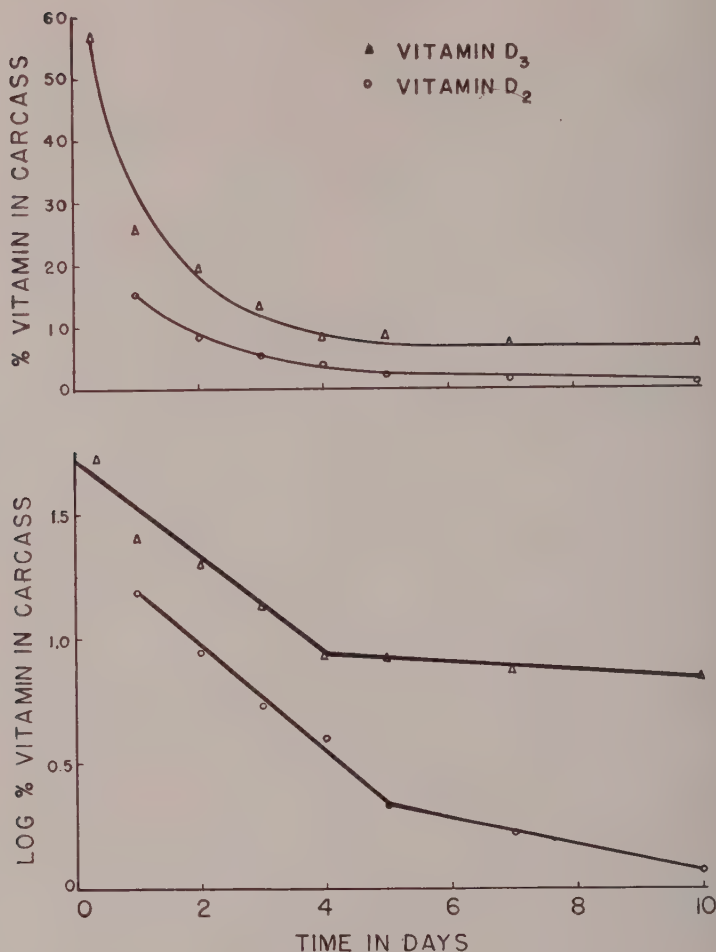


FIG. 1.
Metabolism of vitamin D in the chick. Percentage and log % of oral dose remaining in the body at various time intervals after administration.

the dose which was given. However, in the case of vitamin D₂ the recovery was not quantitative. It was never possible to recover more than 10 to 15% of the dose given from the carcasses of the chicks, even when they were killed at 8 or 16 hr after medication. This amount, together with the 52% found in the feces, leaves one-third of the dosage unaccounted for. It would be logical to suppose that the missing fraction is inactivated in the digestive tract.

The curves which result, when per cent vitamin recovered from carcasses is plotted against time after medication, reveal little more than the fact that there remains at any

given time a much larger residue of the vitamin D₃ than of the vitamin D₂. In comparison with the dose given there remains on the tenth day about 5 times as much of the vitamin D₃ as of the vitamin D₂. The kinetics of the disappearance of the vitamin D activity becomes more apparent when the data are plotted as log percentage-vitamin-remaining in carcasses against time after medication. The curves do not seem to represent simple first-order reactions since each is apparently made up of two distinct parts or phases. In the first phase the destruction of the vitamins is quite rapid and parallel, with a half-life period for both of about 1.4 days. In the second the

destruction is much slower, and a difference between the 2 vitamins is apparent. The half-life period of vitamin D₂ during this phase is 2.7 days, while the half-life period for vitamin D₃ is 3.9 days. The destruction of vitamin D₂ is, therefore, proceeding at a rate exceeding that of vitamin D₃ by approximately 50%.

Summary. The metabolism of large (hypercalcemic) dosages of vitamins D₂ and D₃ has been studied in the chick. The chick evidently does not absorb vitamin D₂ as well as it does vitamin D₃, at least in the doses given; a larger proportion of it is found in the feces,

and less is found in the carcasses. The amounts of vitamin D₃ found in carcasses and feces account for all of the dosage given, but about 35% of the vitamin D₂ cannot be accounted for. Analysis of the data indicated that there are two phases of inactivation of the vitamins. In the first both are inactivated at about the same rate, but in the second phase the rate of destruction of vitamin D₂ is about 50% greater. The generally more rapid inactivation of vitamin D₂ in the tissues may have a bearing on the fact that it is less effective in the chick than is vitamin D₃.

14933

Supplemental Use of Ergotamine in Eliciting Transient Disturbances of Rhythm in Acute Rheumatic Fever.

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The diagnosis of acute rheumatic fever has frequently been found, among soldiers, to be less dependent on the elicitation of significant cardiac murmurs than on the registration of transient aberrations in sequential electrocardiographic curves.¹ For the most part, these aberrations have consisted of various types of disturbances in rhythm due to a temporary hypervagotonic state excited by the acute rheumatic process.^{1,2} However, in a significant number of instances, such electrocardiographic alterations, presumably because of their very brief duration¹ may never be apparent even in repeated tracings. Therefore, in an attempt to improve the usefulness of the electrocardiograph in such circumstances, the author undertook a clinical study in which this diagnostic aid was employed, following the administration of a sympatholytic preparation such as ergotamine tartrate (Gynergen). The results obtained by this method would seem to justify this brief preliminary report concerning the utility of such a simple supplementary procedure.

Material and Method. The observations

were made in a random group of 8 soldiers suffering from acute migratory polyarthritis. In most instances no clinical evidence of cardiac disease was discernible. A routine electrocardiogram, in all cases, was obtained with the patient in the recumbent position shortly after his admission to the hospital. If the tracing revealed no significant changes, a control curve was obtained immediately preceding the intravenous administration of 0.5 mg of ergotamine tartrate, followed by comparable sequential records at 30 and 60 min intervals after the exhibition of the drug. Each of the 8 patients was under close medical surveillance during the convalescent stage of his illness and in 5 of them, a similar type of study was repeated after the usual manifestations of activity of the infection had disappeared.

Results. In each of the 8 patients, the initial electrocardiogram, before the administration of the ergotamine tartrate, was within normal limits. In 6 of the cases, comparative tracings obtained after the exhibition of this drug revealed significant disturbances in rhythm of a type usually associated with acute rheumatic fever, and consisted of first degree A-V heart block in 4 instances, second degree

¹ Wendkos, M. H., and Noll, J., *Med. Clin. N. A.*, 1944, **2**, 124.

² Wendkos, M. H., unpublished data.

TABLE I.

Case No.	Age	Date	Electrocardiographic response to ergotamine	Remarks
1	24	2-14-44	Second degree A-V heart block with dropped beats	Rapid sedimentation time. Joint pains present.
1	24	3-25	Some slowing of sinus rate	Normal sedimentation time. Joint pains absent.
2	21	1-24	Nodal rhythm with first degree A-V heart block	Rapid sedimentation time. Acute polyarthritis.
2	21	3-1	Some slowing of sinus rate	Normal sedimentation time. Joint pains absent.
3	19	12-10-43	First degree A-V heart block (PR—0.28)	Rapid sedimentation time. Acute polyarthritis.
3	19	3-10	Some slowing of sinus rate	Normal sedimentation time. Joint symptoms absent.
4	21	3-12-44	First degree A-V heart block (PR—0.40)	Rapid sedimentation time. Joint pains both shoulders.
4	21	3-23	Some slowing of sinus rate	Sedimentation time reduced. Joint pains absent.
5	19	3-12	First degree A-V heart block (PR—0.32)	Sedimentation time rapid. Acute polyarthritis.
5	19	3-18	Some slowing of sinus rate	Sedimentation time reduced. Arthritis completely disappeared.
6	34	1-3	First degree A-V heart block (PR—0.30)	Sedimentation time rapid. Acute polyarthritis, some persistent deformity, swelling in small joints of hand.

A-V heart block with dropped beats in one instance, and nodal rhythm with first degree A-V heart block in another (Table I). These ergotamine-induced changes were of brief duration and generally disappeared within 60 min after the drug was administered. The maximum effect usually was noted within 30 min after its use. No untoward reactions except for occasional and transient nausea and headache were observed. In the 5 individuals in whom this testing method was employed for comparative purposes after the active phase of the disease had disappeared, the effect was similar to that which occurs in the normal subject (Fig. 1) and consisted of a slight slowing of the sinus rate (Table I). This response was in marked contrast to the significant disturbances of rhythm which had been provoked by the ergotamine during the early stage of the acute rheumatic process. This phenomenon is well illustrated in a typical instance by Fig. 2 and 3.

Illustrative Case. A 24-year-old white male was admitted to the hospital because of aching in various joints of his body. He first became ill one month previously, the onset of the illness being acute and characterized by chills, fever and sore throat. Four days later he developed redness and swelling in both hands and in both feet. These arthritic symptoms, as well as the chills and fever, subsided 3 days later. However, after an interval of well-being for several days, he developed stiffness and pain in the lower back, both knees, both elbows, both wrists, and the fingers of both hands. The stiffness and pain in these joints became progressively more severe so he was eventually admitted to the medical ward with a tentative diagnosis of rheumatoid arthritis. The patient gave no history of antecedent rheumatic infection, and aside from seasonal attacks of hay fever, he had always been in good health. The physical examination, including careful attention to the heart, revealed no significant pathology. Passive movement in all the joints was unrestricted, and no arthritic swelling or redness was noted. The day following his hospital admission, the erythrocyte sedimentation rate was estimated to be 78 mm in one hour (normal—15 mm), and his electrocardiogram was not significantly altered (Fig. 2A).

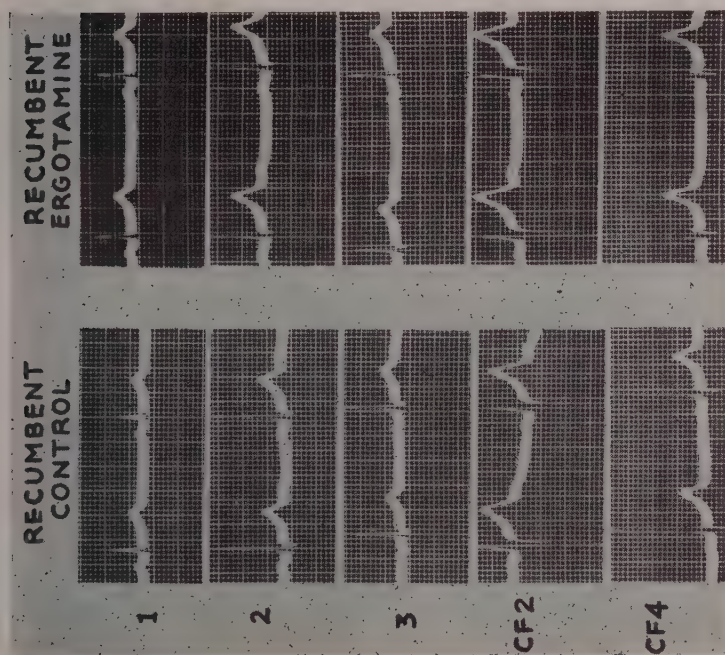


Fig. 1.
The electrocardiographic registration of the parasympathetic effect of ergotamine tartrate in a normal subject; The response is characterized by a slowing of the sinus rate and by a slight increase in amplitude of the T waves in each lead.

However, at that time, following the administration of ergotamine tartrate, a second degree A-V heart block with dropped beats was induced (Fig. 2B). His subsequent clinical course was uneventful and within 6 weeks his sedimentation time had dropped to normal and his joint symptoms had improved. During his entire period of hospitalization, no abnormalities of the heart were revealed in the repeated clinical examinations, and several electrocardiograms obtained at weekly intervals demonstrated no aberrations. During the convalescent stage of his illness, after all collateral evidences of persistent activity of the infection had disappeared, the procedure employing ergotamine tartrate was repeated, and no effect except for some slowing of the sinus rate was apparent (Fig. 3).

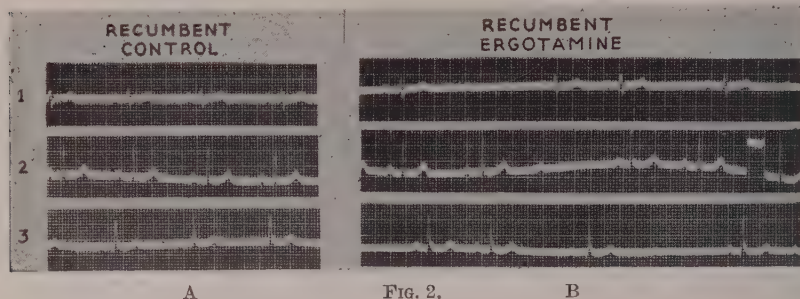
Discussion. Autonomic imbalance with vagal preponderance undoubtedly is an important component of the disturbed physiology associated with the acute rheumatic state, because it has been conclusively demonstrated that a parasympatholytic drug such as atropine can temporarily abolish the first degree A-V heart block^{1,3,4} and other types of rhythm disturbances² which are the chief electrocardio-

graphic expressions of the disease. However, since characteristic features of these disturbances in rhythm are their inconstancy and transient nature, it is likely that a spontaneous variation occurs in the intensity of discharge of the exaggerated cholinergic stimuli in the early stages of rheumatic fever, it being implied that the subordination of a hypervagotonic state below a certain critical level will prevent the registration of the usual electrocardiographic representations of such vegetative dysfunction. Under circumstances in which this "latent" phase of vagal preponderance conceivably is present in individuals suffering from acute rheumatism, the suppression of activity of the normal antagonist of the vagus by the use of a sympatholytic preparation such as ergotamine tartrate⁵ would be expected to provoke, in their electrocardiograms, vagal effects, akin to, but not necessarily identical with, the precordial lead T wave alterations

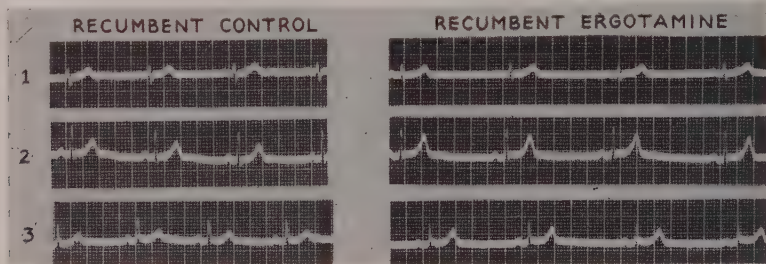
³ Bruenn, H. G., *Am. Heart J.*, 1937, **13**, 413.

⁴ Keith, J. D., *Quart. J. Med.*, 1938, **7**, 29.

⁵ Goodman, L., and Gilman, A., *The Pharmacological Basis of Therapeutics*, New York, Macmillan Company, 1941.



The electrocardiographic registration of the parasympatheticomimetic effect of ergotamine tartrate in an individual during the early active phase of acute rheumatic fever: The effect primarily is upon the A-V node, resulting in the development of a second-degree heart block.



The electrocardiographic registration of the parasympatheticomimetic effect of ergotamine tartrate in the same individual as in Fig. 2, during the subacute phase of rheumatic fever: The effect is similar to that which occurs in a normal subject. (See Fig. 1.)

produced by this drug in emotionally unstable individuals, when a "latent" stage of "cholinergia" exists.^{2,6} Actually when the initial record was normal significant disturbances in rhythm were usually elicited by this method in the relatively few cases of acute rheumatic infection in which it was employed. (Table I)

Even though the series is small, the results seem to be significant inasmuch as studies, which are based on experiments in large numbers of individuals in whom no imbalance of the autonomic nervous system would be expected to occur, have demonstrated^{2,7,8} that the only physiological effects of ergotamine tartrate upon the human electrocardiogram consist of a slowing of the sinus rate and a slight increase in the amplitude of the T waves (Fig. 1). Since this effect is not unlike that which occurs in an individual who is in the

subacute stage of a rheumatic infection (Fig. 3) in contrast to the response to the same agent in the same subject when collateral evidences of active infection existed (Fig. 2), it is probably reasonable to suppose that this testing method, besides assisting in the diagnosis of acute rheumatic fever, may also be found useful in helping to decide when recovery is beginning to occur.

Conclusions. 1. It is suggested, from a limited experience with the method, that the supplemental administration of ergotamine tartrate may be useful in increasing the precision of the electrocardiograph as a diagnostic aid in acute rheumatic fever. 2. It is considered that the rationale of this procedure depends on the elevation of a subordinated or "latent" hypervagotonia by a suppression of activity of the normal antagonist of the vagus, so that "cholinergigenic" disturbances in rhythm are thus made manifest in the electrocardiogram. 3. The procedure is simple, can be standardized easily and is without any harmful effects.

⁶ Wendkos, M. H., *Am. Heart J.*, 1944, **28**, 549.

⁷ Nordenfeldt, O., *Acta. med. Scandinav.*, 1941, Supp., CXIX.

⁸ Hartwell, A. S., Burrett, J. B., Graybiel, A., and White, P. D., *J. Clin. Invest.*, 1942, **31**, 409.

The Plasma of Developing Chick and Pig Embryos.

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(Introduced by E. T. Engle.)

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Several extensive studies¹⁻⁴ have been made on the serum proteins from a number of animal species after birth, but no work has so far been reported on the changes occurring in the blood proteins during embryonic development. In order to learn more about the origin and physiological development of the plasma proteins we have obtained electrophoresis, ultracentrifuge, and some chemical data on sera and plasmas from developing chick and pig embryos. By all methods of analyses the embryonic and fetal plasma differs widely from that of the adult, and electrophoretic analyses show rapid changes during development.

Chick Plasma. White leghorn eggs were incubated from 8 to 21 days at 37°. Blood from a large number of embryos of the same age was taken directly from the larger arteries

of the living embryos with a tuberculin syringe and then pooled.

All electrophoresis, ultracentrifuge, and diffusion measurements were made after dialysis in viscose casings (average pore diameter $2 - 3 \times 10^{-7}$ cm) at 0° to 4° against a buffer containing 0.02M sodium phosphate and 0.15M NaCl. The electrophoretic data obtained on the chick embryo plasma are given in Table I and the corresponding electrophoretic patterns are presented in Fig. 1. For comparison, similar data on egg white, 3-day-old chick and adult chicken plasmas are also given. The components are classified roughly according to their mobilities and are numbered from 1 to 5 in order of decreasing mobility. Component 3, which has a mobility approximating that of β -globulin in the serum of the adults of most species, is predominant in the earlier embryos, but gradually recedes throughout the development. Component 1 does not become appreciable until about the eleventh day and appears to reach maximum at about the time of hatching (data and patterns not shown). Components 2 and 4, which appear after 16 to 18 days of incubation, varied considerably in blood from fetuses of the same age as well as in newly hatched and adult chickens, but were large in all cases after hatching.

Several samples of whole plasma from the developing chick embryos and a few electrophoretically separated fractions have been studied in the analytical ultracentrifuge (Table II). In the unfractionated chick plasma only a single component was observed as in Fig. 2A, B and C. This component has a sedimentation constant different from either albumin (4.5 Svedberg units) or the bulk of the globulin (7.7 Svedberg units) found in the adult of the same species. The sedimentation constant seemed to decrease slightly with in-

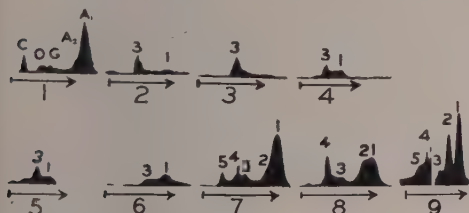


FIG. 1.

Electrophoresis patterns of (1) fresh egg white, (2-8) chick embryo, and (9) adult chicken plasma. Pattern numbers correspond to numbers in first column of Table I. In pattern (1) A₁, A₂ = albumin, G = globulins, O = ovomucoid, C = conalbumin.

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1 Utheim, K., *Am. J. Dis. Children*, 1920, **20**, 366.

2 Howe, P. E., *J. Biol. Chem.*, 1922, **53**, 479.

3 Jameson, E., Alvarez-Tostado, C., and Sorter, H. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 163.

4 Pedersen, K. O., *The Svedberg Anniversary Volume*, p. 490, Upsala, 1944.

TABLE I.
 Electrophoretic Fractionation of Chick Embryo Plasma.*

No.	Dilution†	Mobility $\times 10^5$ cm ² /volt sec						Composition %				
		A ₁	A ₂	G	O	C		A ₁	A ₂	G	O	C
1	Egg white	1:10	4.7	4.2	2.6	1.9	1.0	60	10	7	8	15
	Embryo age days	Dilution†	Mobility $\times 10^5$ cm ² /volt sec					Composition %				
			1	2	3	4	5	1	2	3	4	5
2	8	1:2	5.8		3.4			10		90		
3	9	1:3	5.4		3.4			15		85		
4	10	2:3	5.8		3.8	2.2		25		70	5	
5	11	1:2.2	5.5		3.5	1.7		30		60	10	
6	13	1:3	5.5		3.9	2.3		60		35	5	
7	17	1:1	5.5	4.7	2.8	2.4	1.6	55	10	8	13	14
8	+3	1:2.4	5.1	4.7	2.9	1.9	1.5	35	25	10	25	5
9	Adult	1:4	5.3	4.4	3.5	2.5		40	25	5	20	10

* Buffer: 0.02M sodium phosphate, 0.15M NaCl, pH 7.4.

† Ratio of plasma volume to volume of solution; thus 1:1 means undiluted.

+3 = 3 days after hatching.

 TABLE II.
 Sedimentation Constants of Chick Embryo Plasma.

Embryo age days	Sedimentation constant*
9	4.2
10	4.2
11	4.2
12	4.0
13	3.9
15	4.1
16	4.0
21	3.6
21	3.6
21 slow	4.0
Adult fast	4.5
Adult slow	7.7 19.0†

* In Svedberg units = 10^{-13} cm/sec per unit field.

† See indicated pattern of Fig. 2.

‡ Trace only.

creasing age of the embryo but did not vary appreciably with concentration (0.6% maximum concentration used). Components 3, 4 and 5 (see Fig. 1) were electrophoretically separated from the plasma of a newly hatched

chick and were found to contain the component having $S_{20} = 4.0$ and a trace of material, possibly masked by the predominant component in whole plasma, having a lower sedimentation constant (see Fig. 2D).

Diffusion constants were determined on 2 pooled plasma samples from embryos incubated 12 and 21 days and were calculated to be 1.6 and 2.5×10^{-7} cm²/sec respectively, at 20°. Diffusion measurements on serum (no anti-coagulant added to the blood) from 11-day embryos gave $D_{20} = 1.8 \times 10^{-7}$ cm²/sec. No difference in the sedimentation patterns of serum and plasma was detected. These values of S_{20} and D_{20} would indicate a molecular weight of between 200,000 and 300,000, the molecules having a high frictional ratio ($f/f_0 = 3.0$). The partial specific volume is as yet undetermined but preliminary measurements indicate that it is not greatly different from most serum proteins, *i.e.*, *ca.* 0.75.

 TABLE III.
 Electrophoretic Fractionation of Pig Embryo Plasma.*

No.	Embryo length mm	Dilution†	Mobilities $\times 10^5$ cm ² /volt sec						Composition %					
			1	2	3	4	5	6	1	2	3	4	5	6
1	30 P‡	1:4	6.2	5.0	3.9		1.9		10	50	15		25	
2	130 P	1:2	5.3	4.5	3.5	2.5			35	35	20	10		
3	160 P	1:2	5.1	4.3	3.8	2.7			45	40	10	5		
4	180 S	1:1	4.7	3.5		2.7	1.6		45	40		10	5	
5	250 S	1:1	4.5	3.3		2.4	1.7		55	35		7	3	
6	300 P	1:2	4.6	3.3		2.5		0.9	50	30		5		15
7	Adult S	1:4	4.8	3.3		2.4		1.2	50	15		15		20

* Buffer: 0.02M sodium phosphate, 0.15M NaCl, pH 7.4.

† Ratio of volume of plasma or serum to volume of solution, thus 1:1 means undiluted.

‡ P = plasma, S = serum.

TABLE IV.
Sedimentation Constants of Pig Embryo Plasma.

Embryo length mm		Sedimentation constant*	
30	A†	3.9	
95		3.7	
180		3.8	
180 slow		3.7	
250	B	3.5	
250 fast	C	3.4	
250 slow	D	2.9	5.9

* In Svedberg units = 10^{-13} cm/sec per unit field.

† See indicated pattern of Fig. 4.

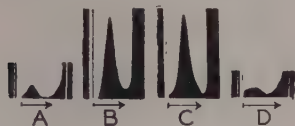


Fig. 2.

Ultracentrifuge patterns of chick embryo plasma.
(See Table II.)

After dialysis against 0.9% NaCl a sample of serum from 13-day-old embryos was found to contain 0.66 mg N/ml of original serum. The protein was dialyzed against H_2O , lyophilized and dried in vacuum to constant weight at room temperature over P_2O_5 . 6.0% N by Kjeldahl and 2.0% ash were found.[†] Several samples from embryos of various ages were analyzed for non-diffusible carbohydrate by a colorimetric⁵ method and found to contain several times as much per unit N as was found in adult chicken serum.[‡] These analyses were made after dialysis against either phosphate-NaCl buffer or H_2O . Electrophoretically separated components both contained carbohydrate, but considerably more was associated with the faster fraction. A great part of the sugar is evidently bound with the protein. The nature of this carbohydrate is under investigation by Dr. Dische and will be reported later.

Pig Plasma. Umbilical cord or heart blood was collected from an entire litter of pig embryos obtained from freshly slaughtered sows.[§]

⁵ Dische, A., and Popper, H., *Biochem. Z.*, 1926, **175**, 371.

† We are indebted to Dr. Manfred Mayer for these determinations.

‡ We are indebted to Dr. Zacharias Dische for these measurements.

§ Kindly furnished by Figgie and Hutwelker Co., slaughterers, New York, N. Y.

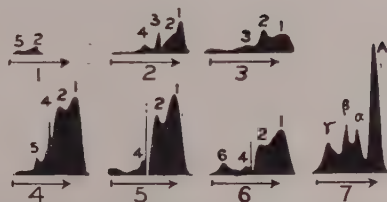


FIG. 3.

Electrophoresis patterns of pig embryo plasma and sera. Pattern numbers correspond to numbers in first column of Table III.

To some samples enough citrate was added to prevent coagulation. The electrophoretic data and patterns are given in Table III and Fig. 3. In the plasma from 30 mm (measured from snout to tail root) embryos, components 1 and 3 are only indicated, component 2 being the predominant one. Component 1 increases, however, throughout the embryonic and fetal development, but component 2 remains an appreciable proportion of the total even in the 300 mm fetus. The body length at parturition is probably between 350 and 400 mm.

Ultracentrifugal analyses of unfractionated plasma and sera indicated only a single component (Table IV, Fig. 4) whose sedimentation constant ranged from 3.9 to 3.5 Svedberg units. Components 1 and 2 (pattern 5 of Fig. 3) separated electrophoretically were homogeneous in the ultracentrifuge (Fig. 4C),[‡] having $S_{20} = 3.4$, but the slower fractions (components 4 and 5) revealed two components (Fig. 4D) with $S_{20} = 2.9$ and 5.9 (in whole serum these were possibly masked by the main component). The component having a sedimentation constant of 2.9 Svedberg units is probably the one described by Pedersen.⁴

Diffusion constants were determined on unfractionated sera from 180 and 250 mm pig fetuses and found to be 5.9 and 5.3×10^{-7} cm²/sec, respectively. These figures are very different from those obtained on the plasma

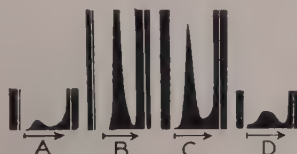


FIG. 4.

Ultracentrifuge patterns of pig embryo plasma.
(See Table IV.)

from chick embryos of various ages, and lead to a molecular weight of about 60,000.

Total solids in serum samples from 180 and 250 mm pigs were found to be 17.2 and 17.4 mg/ml respectively after dialysis against H₂O and lyophilization. Nitrogen determinations yielded 12.5% and 13.2% respectively, values which did not change measurably after a portion of the solids were refluxed for 24 hr with alcohol-ether under N, indicating little or no substances soluble in organic solvents. Three small aliquots (1 to 3 mg each) from each sample of lyophilized solid were analyzed for phosphorus but were found to contain only traces.^{||}

The ratio of carbohydrate to nitrogen in the pig embryo serum, like that in the chick, was high, being 2 to 3 times that in the serum of adult swine. Whether or not all of this is bound to the protein, further investigation will show.

A correlation of these findings with the

^{||} We are greatly indebted to Miss Helen Fabricant for these analyses.

morphogenesis and organogenesis of the embryos is being made.

Summary. Electrophoresis patterns show rapid changes in the plasma of developing chick and pig embryos. In the ultra-centrifuge only a single component is apparent in the unfractionated plasma. After electrophoretic fractionation of the plasma or sera from older fetuses, traces of other components are revealed.

Diffusion and sedimentation constants of 11-day chick embryo serum would indicate a molecular weight of more than 200,000. The diffusion rate of the plasma from older chick fetuses was greater. Pig fetus plasma had a much larger diffusion constant, indicating a molecular weight of approximately 60,000 for the bulk of the plasma protein from 180 and 250 mm fetuses.

Thirteen-day-old chick serum protein contained 6.0% N and 20 to 30% carbohydrate, whereas 180 mm pig embryo serum protein contained 12.5% N and 15 to 20% carbohydrate.

14935

Simple Method of Preparing Potent Blood Grouping Sera.*

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and the Brooklyn Cancer Institute.*

The properties A and B are antigenic for human beings whose erythrocytes lack the corresponding agglutinogens, as was demonstrated by observations made following unintentional transfusions of blood of an incompatible group.^{1,2} Aubert, Boorman, and Dodd³ observed a similar increase in isoagglutinin titer in patients transfused with serum

prepared from the blood of donors belonging to an incompatible group. Wiener⁴ applied this observation as a method of preparing high-titered blood grouping sera, by immunizing professional blood donors with transfusions of dried pooled plasma (Sharp and Dohme) reconstituted with distilled water.[†] Satisfac-

* Wiener, A. S., *J. Immunol.*, 1941, **41**, 181.

³ Aubert, E. F., Boorman, K. E., and Dodd, B. E., *J. Path. and Bact.*, 1942, **54**, 89.

⁴ Wiener, A. S., unpublished observations.

[†] Based on these findings, individuals of group O who have previously received plasma transfusions should not be used as universal donors, unless it is proved by careful titration tests that their sera do not contain dangerously high concentrations of isoagglutinins.

* The work in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Jewish Hospital of Brooklyn. The work was also aided in part by a grant from the United Hospital Fund of New York City.

¹ Wiener, A. S., Orenland, B. H., Hyman, M. A., and Samwick, A. A., *Am. J. Clin. Path.*, 1941, **11**, 102.

tory typing sera were obtained from about half of the donors injected.

A simpler method was developed by Witebsky *et al.*⁵ entailing the use of solutions containing a mixture of purified AB substance (isolated from horse stomach) and A substance (isolated from hog stomach). As little as 0.1 cc of the solution containing 3 to 5 mg of AB substance and 5 to 10 mg of A substance in 10 cc of normal saline solution, when injected intravenously induced a marked increase in the isoagglutinin titer. Moreover, a group O individual injected intramuscularly with 1 cc of the solution showed a 100-fold increase in both isoagglutinins anti-A and anti-B. Wiener⁶ has confirmed this report of Witebsky *et al.* and found that intramuscular injections gave as satisfactory results as intravenous injections of the purified group substances.

The chief disadvantage of the method of Witebsky *et al.* is that the purified substances are not generally available. There is also a theoretical possibility that improperly prepared material may contain traces of animal protein and give rise to foreign protein reactions in rare hypersensitive individuals. Accordingly, the purpose of the present report is to describe a new, simple and safe method of preparing immune high-titered sera, that can be carried out by any competent worker interested in blood grouping.

The new method of preparing high-titered grouping sera is similar in principle to that of Witebsky *et al.*, but entails the use of solutions of A and B substances derived from human saliva. Blood donors are immunized by intramuscular injections of 1 cc of the partially purified saliva, diluted 1:5 with saline solution, and the donors are bled 2 weeks or longer after the injection, provided their serum shows a satisfactory increase in isoagglutinin titer.

The saliva used by us for the injection was processed as follows. The saliva was autoclaved at 15 lb pressure for 15 min, and the coagulated protein and other insoluble material were then easily removed by centrifuging. The supernatant opalescent fluid was mixed with

4 parts of sterile saline solution, and the diluted saliva was distributed among a number of sterile vials of convenient size. To insure sterility the material was autoclaved a second time in the vials which were then closed with sterile rubber caps. The group substances are apparently not damaged by the processing because the inhibition titer of the "purified" saliva was approximately $\frac{1}{5}$ the titer of the original saliva, as expected from the dilution. Incidentally, the use of saliva is advantageous not only because of the ready availability of the material, but also because it excludes the possibility of sensitization to foreign proteins and in addition makes it possible to prepare solutions of the A and B substances separately. Of course one must be certain that the saliva is collected from secretors, but this is no serious problem because 85% of all individuals are secretors. It should be mentioned that if the saliva cannot be processed immediately after collection it should be placed in boiling water for about 10 min in order to destroy the blood group enzymes, and then stored in the refrigerator until processing can be carried out.

Titration experiments showed that the inhibition titer of processed A saliva is about equal to or slightly lower than that of Witebsky's blood group solutions, while processed B saliva is about 4 times as active.

With regard to the antigenic activity of these preparations the following experiment can be cited as an example. Five group O and 3 group A patients were given intramuscular injections of 1 cc of processed group B saliva. After 12 days blood samples were collected and the isoagglutinin titers compared with the original titers of blood taken before the injections. As can be seen from Table I the great majority of the patients responded to the injection with rises in isoagglutinin titers ranging up to 10 times the original value. In some of the group O patients the anti-A titer rose as well as the anti-B titer even though the material injected contained no A substance. This may be explained by postulating the presence in A and B substances of a common property C capable of stimulating the production of specific anti-C isoagglutinins.⁷ Or one could assume that properties A and B have certain similarities in chemical structure and

⁵Witebsky, E., Klendshoj, N. C., and McNeil, C., *PROC. SOC. EXP. BIOL. AND MED.*, 1944, **55**, 167.

⁶Wiener, A. S., unpublished observations.

TABLE I.
Antigenic Effect of Intramuscular Injections of Small Amounts of Autoclaved Group B Saliva.

Patient No.	Blood Group	Initial titer of serum for		Titer after saliva injections	
		A ₂	B	A ₂	B
1	O	6	2	45	60
2	O	6	6	50	90
3	O	2	2	6	4
4	A	0	50	0	400
5	O	1½	3	6	60
6	A	0	7	0	12
7	A	0	75	0	90
8	O	3	25	1½	90

that the cross reactions are due to antibodies of low specificity.^{8,9}

It may be mentioned that saliva processed as described in this paper is more satisfactory for inhibiting anti-A and anti-B isoagglutinins in human anti-Rh sera than pooled, boiled saliva as originally recommended.¹⁰

Comment. According to the classic concept, the group substances owe their specificity to carbohydrate structures, and in the erythrocytes these are supposed to be conjugated with lipids and/or proteins, which confer on the agglutinogens their antigenicity.⁷ In the secretions, the group substances are supposed to be present in a soluble form, as haptens free of proteins and lipids and therefore non-antigenic. Actually, our observations cited in this paper refute these ideas. The intramuscular injections of relatively minute quantities of

saliva have been shown to stimulate substantial rises in isoagglutinin titer. On the other hand, injections of comparable or larger amounts of erythrocytes by the intramuscular route appear to have little or no stimulating effect. In fact, Wiener¹¹ has injected substantial amounts of red cell stromata intramuscularly without producing any appreciable change in isoagglutinin titer. Obviously, our concepts concerning the nature of the group substances in erythrocytes and secretions will have to be revised.

Witebsky's investigations have demonstrated the feasibility of preparing large quantities of purified A and B group substances from animal sources, and such material will undoubtedly soon become generally available. The A and B substances from human saliva will hardly replace the excellent product from animal sources, but may serve as a temporary stop-gap in emergencies wherever the commercially prepared A and B substances are difficult or impossible to obtain.

Conclusions. The intramuscular injection of small amounts of autoclaved saliva (0.2 cc) from secretors of groups A and B often stimulates a considerable increase in the isoagglutinin titer. This method simplifies the large-scale production of potent blood grouping sera.

⁷ Wiener, A. S., *Blood Groups and Transfusions*, 3rd ed., p. 293, C. C. Thomas, Springfield, Ill., 1943.

⁸ Wiener, A. S., and Karowe, H. E., *J. Immunol.*, 1944, **49**, 51.

⁹ Boyd, W. C., and Warshaver, E. R., *J. Immunol.*, 1945, **50**, 101.

¹⁰ Wiener, A. S., and Forer, S., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 215.

¹¹ Belkin, R. B., and Wiener, A. S., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 214.

14936 P

Presence of Granules Resembling Elementary Bodies in Yolk Cells of Normal Eggs.

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Blood from a case of anemia associated with the presence in the red blood cells of unidentified siderophilic granules¹ was inoculated onto the chorio-allantoic membranes and into the yolk sac of developing chick embryos. Sections were prepared from the membranes and, in Giemsa-stained preparations, minute purplish blue granules were found in great numbers in the cytoplasm of the yolk cells. In order to reach a conclusion as to their possible identity with the intra-erythrocytic bodies, control eggs were inoculated with saline, normal blood, influenza virus, and other ma-

terials; and finally, uninoculated eggs in various stages of development were examined. It was surprising to find in all eggs, treated or untreated, after the 11th or 12th day, increasing numbers of these granules (Fig. 1). Because of their close resemblance to described elementary bodies of certain viruses, and because we have found no reference to their occurrence in uninoculated eggs, we desire to give a brief account of our observations.

Paraffin sections of the membranes were prepared from more than 200 eggs fixed in 10% formalin, Zenker's fluid without acetic acid and

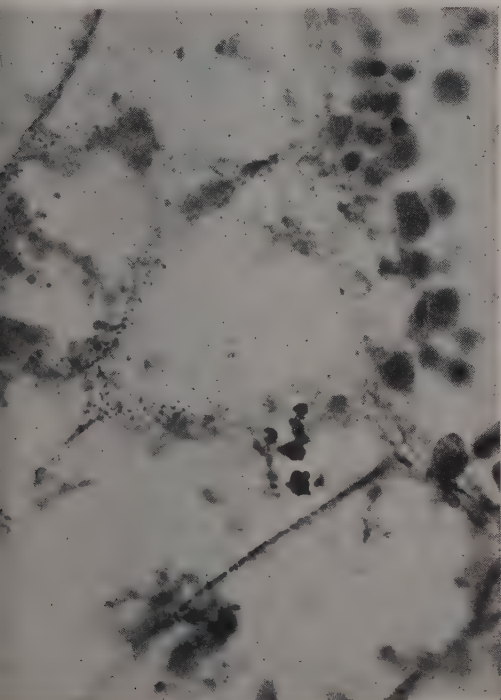


FIG. 1.

Yolk sac. Giemsa stain. $\times 1050$. Egg inoculated on day of incubation with blood from patient K. Taken for examination on 14th day.

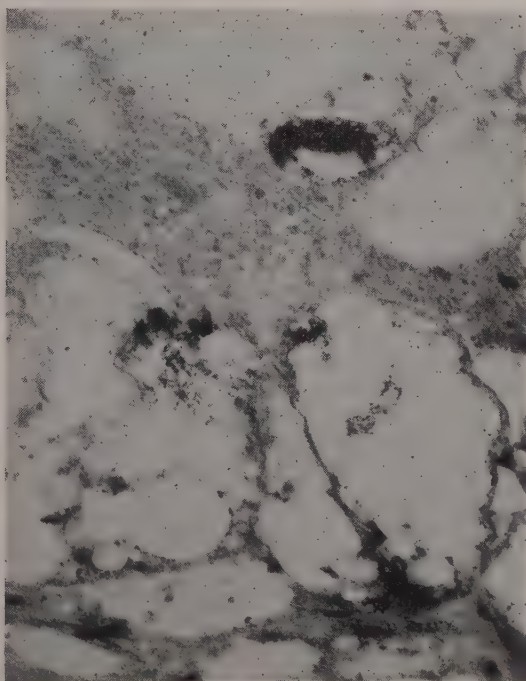


FIG. 2.

Yolk sac, normal uninoculated egg. 17 days incubation. Giemsa stain. $\times 1050$.

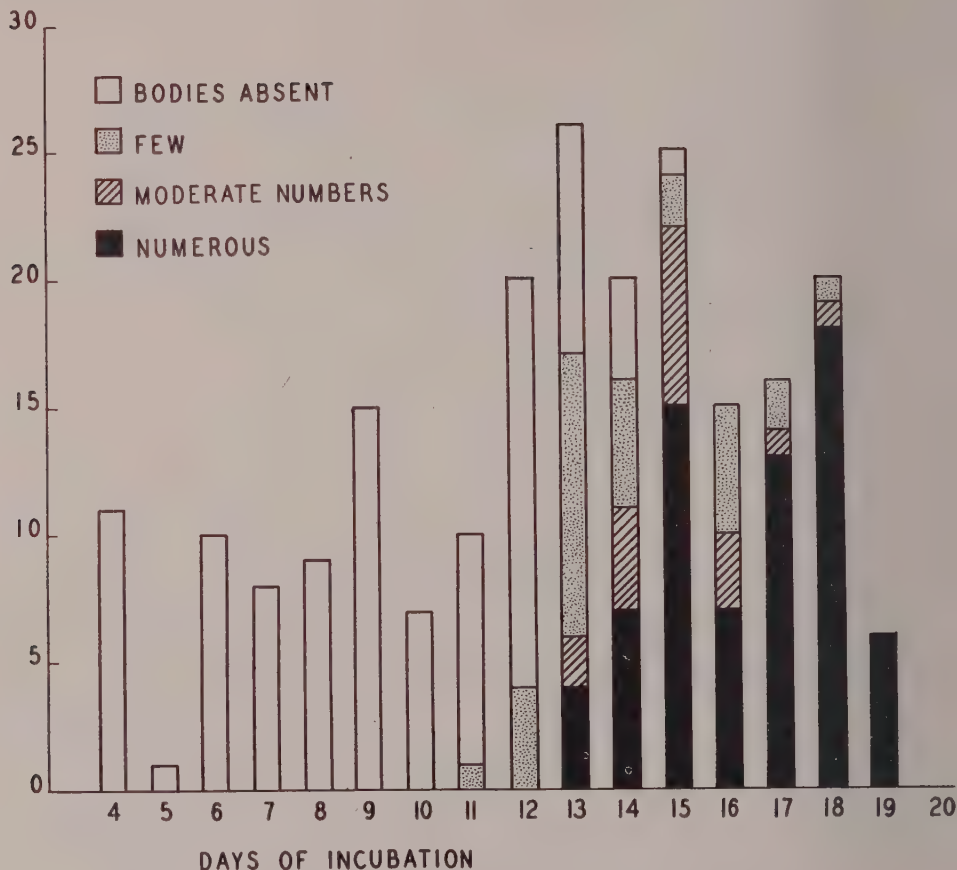
NO OF EGGS.
EXAMINED

CHART 1.

Bouin's fluid.* They were stained with Giemsa, eosin-methylene blue, Noble's stain, and by Nyka's rickettsial stain,² and the bodies were readily demonstrable by all these methods. The bodies were found within the yolk cells as scattered individuals, or as large agglomerations. Their size varied from a fraction of a micron up to a maximum of 1

or 2 μ . The staining also varied somewhat, the minute forms taking a reddish purple stain with Giemsa and the larger forms a more intense blue. Diploid forms were repeatedly seen and, occasionally, short delicate rod shapes. The bodies were often aligned in a single row along the wall of the cells; commonly large colonial aggregations occupied the shreddy protoplasmic processes which projected from the cell wall. (Fig. 1, 2, 3). They were rarely found among the extra-cellular yolk granules, and among the developing blood cells in the sinuses. It is apparent from the accompanying chart that the bodies first make their appearance about the 11th or 12th day of incubation, and from this time on are found

* The eggs used in this study were White Leg-horns, obtained from the Shamrock Poultry Farms, New Brunswick, N. J.

¹ Pappenheimer, A. M., Thompson, W. P., Parker, D., and Smith, K. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1944, **56**, 145.

² Nyka, W., *J. Path. and Bact.*, 1944, **56**, 264.

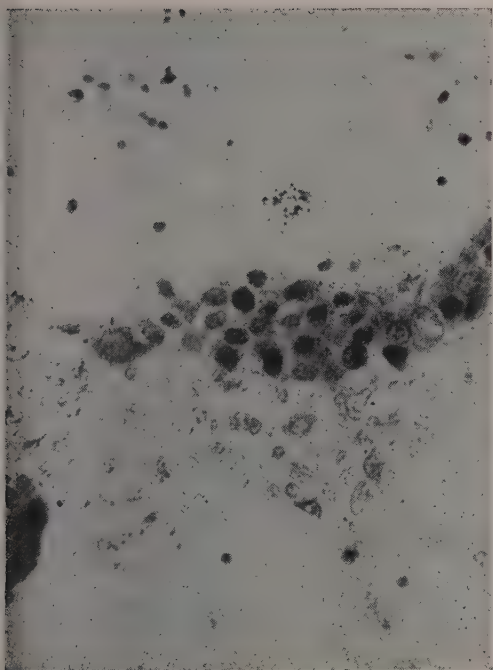


FIG. 3.

Yolk sac, normal uninoculated egg. 18 days' incubation. Nyka's fuchsin-methyl violet stain. $\times 1050$.

in increasing numbers up to the 19th day.

That the bodies are due to staining artefacts

seems highly improbable, since they may be demonstrated after different methods of fixation, with different staining techniques, and have a well-defined morphology and sharp outline. Their absence in the yolk cells before the 10th day is a further argument against their being artefacts. There remain two possible interpretations of their nature:

1. They are cytoplasmic constituents of normal yolk cells, appearing in the course of their evolution. Against this viewpoint is the fact that they are not present in all cells and indeed, during the first few days after their appearance, may be scant in number and require prolonged search for their detection.

2. They are elementary virus bodies or psittacosis-like micro-organisms, present as harmless commensals in the developing yolk cells. The finding of diploid forms and the progressive increase in the numbers of the bodies suggests active multiplication. Further experiments are obviously indicated before drawing conclusions as to the nature of these bodies.

Since the submission of this proof, identical bodies have been found in the yolk cells of eggs from Barred Plymouth Rocks and Rhode Island Reds sent to us from the Storrs Agricultural Experiment Station through the courtesy of Dr. Erwin Jungherr.

14937

Adaptation of Influenza Virus to Heat.*

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Bacteria have been adapted many times to growth or survival under a number of physical conditions, including heat, which are entirely foreign to any environmental circumstances

encountered in normal multiplication. Due to numerous technical difficulties, very few similar studies with viruses have been carried out.

Armstrong¹ was able to select a heat-resistant strain of vaccinia virus by passing samples of virus which withstood the longest storage periods at 37°C. At the end of the 63rd transfer, the virus withstood that temp-

* This investigation was aided in part by the Commission on Influenza Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army, Washington, D.C.

¹ Armstrong, Charles, *Pub. Health Rep.*, 1929, **44**, 1183.

erature for 33 days. Enders and Pearson² were unable to cultivate the Melbourne strain of influenza A virus at 41°C, and could not infect day old chicks with that virus, although they could recover virus from their lungs 19 hours after inoculation. They believed that the body temperature of the chick (41°C) might be a limiting factor in their resistance.

A number of investigators (Fairbrother,³ Henle and Henle,⁴ and Hirst^{5,6}) have determined the temperatures necessary to inactivate the influenza virus. These workers found that temperatures of 56°C to 57°C for 30 to 45 min were necessary for this inactivation. Determination of virus survival was made by mouse infectivity tests, or by the ability of the virus to initiate growth in chorioallantoic sac of embryonated eggs.

In order to test the adaptability of influenza A virus to heat, 2 strains were used, the mouse-adapted PR8* and the IC† strains of influenza A viruses. The PR8 strain was in its 507th mouse passage, while the IC strain was in its 50th egg passage. The latter strain was isolated in Iowa City during the influenza epidemic of December 1943 by inoculation of throat washings into the chorioallantoic sac of embryonated eggs, and had never been in another medium.

The PR8 strain of influenza virus was studied in relation to its adaptability to propagation at 41°C in tissue culture medium and the chorioallantoic sac of embryonated eggs. Tissue culture medium was prepared in the usual manner under aseptic conditions, using minced 9- to 10-day chick embryo tissue suspended in 4.5 ml of Tyrode's solution placed in 50 ml rubber-stoppered flasks. Such media were stored at 37°C for 1 to 6 days before use. The sterile flasks of tissue culture were inocu-

lated with 0.5 ml of tissue culture fluid containing virus and incubated at 37°C or 41°C for 2 day periods. When storage of infected tissue cultures was necessary, such storage was carried out at refrigerator temperature.

The PR8 strain of influenza A virus grew in tissue culture medium at 41°C, and was carried for 153 passages. Titrations of passages 30 and 91 in mice (Swiss strain) showed active virus present in titers of 10⁻² for mortality and 10⁻³ for lesion production, as contrasted to titers of 10⁻³ for mortality and 10⁻⁴ for lesion production with control virus grown at 37°C. This indicated that growth at the higher temperature, although occurring, was less abundant than at 37°C. The virulence for mice apparently remained unchanged, however, because subcultures at 37°C from the 41°C strain gave titers identical with the controls. Continued passage at 41°C did not give evidence of any adaptation to heat, since growth constantly remained poor. The PR8 strain also grew in the chorioallantoic sac (7 passages) of 12-day embryonated eggs incubated at 41°C. Here, also, growth remained poor, as indicated by mouse infectivity tests. One and 2-day-old chicks were given separately the tissue culture or allantoic fluid containing the 41°C and 37°C strains of influenza virus intranasally under ether anaesthesia. Both strains could be recovered from chick lungs 19 to 24 hr after inoculation but not after 48 hr. No evidence was obtained that the 41°C strain survived any longer in the chick than did the control strain.

Since no evidence of adaptation to heat could be obtained by growth of the PR8 strain of influenza virus at 41°C, an inter-transfer method of heat treatment at higher temperatures was adopted to try to increase the resistance of the virus to heat. For preliminary study of heat shock on the virus, the PR8 strain was grown in tissue culture medium incubated at 37°C for 2 days; 1 ml quantities of the virus-containing medium were transferred to cotton-stoppered conical centrifuge tubes or to sealed vials, and were heated at various temperatures in a constant temperature water bath. This heated virus was then inoculated into fresh tissue culture medium and incubated at 37°C. The test for the presence of active

² Enders, John F., and Pearson, Harold E., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **48**, 143.

³ Fairbrother, R. W., *Lancet*, 1938, **1**, 1269.

⁴ Henle, Werner, and Henle, Gertrude, *J. Bact.*, 1943, **46**, 314.

⁵ Hirst, George K., *J. Exp. Med.*, 1942, **76**, 195.

⁶ Hirst, George K., *J. Exp. Med.*, 1943, **78**, 88.

* Dr. T. Francis, Jr., supplied the PR8 strain of influenza A virus.

† Dr. Wm. M. Hale supplied the Iowa City strain of influenza A virus.

TABLE I.
Survival of PR8 Strain of Influenza Virus in Tissue Culture Medium After Single Heat Treatment.

Temperature °C	Time min	Survival
60	5	0
	7	0
55	15	+
	30	±*
	45	0
	60	0
50	60	+
	120	+
	180	0

* Occasionally no virus survived.

virus was made by mouse inoculation; all mice received 0.1 ml inocula intranasally under ether anaesthesia. Table I shows the results of single heat treatments. Since parallel results were obtained when the virus was heated in centrifuge tubes and sealed vials, the centrifuge tubes were used thereafter because of their greater convenience.

When the PR8 strain was heated for second treatments at temperatures over 50°C for periods of over 15 min, active virus could not be shown in culture following the second heat treatment. This was not surprising, since Henle and Henle^{4,7,8} have shown that inactivated virus exerted a marked inhibitory effect on propagation of active virus. The presence of large amounts of heat-inactivated virus apparently inhibited the growth of whatever small quantities of active virus survived, and the additive effect of a second heating was sufficient to prevent growth entirely. However, by gradually increasing the heat shock period from 15 to 60 min at 50°C between each transfer, the virus was carried for 90 passages. This "adapted" virus was tested for its ability to survive single shock treatment, with results as shown in Table II.

As can be seen from a comparison of the results in Tables I and II, the adapted virus was able to withstand heat shock to a greater degree than the unadapted virus. The adapting temperature, however, could not be increased

over 50°C, since at higher temperatures, even for shorter intervals, the virus was repeatedly lost. This was probably caused by the inhibiting effect of a gradually increasing amount of inactive virus.

Further study of adaptive changes in the influenza virus undergoing heat-adaptation was carried out by comparing the effect of storage at different temperatures on this strain and the unadapted control virus. PR8 strain was incubated in tissue culture medium at 37°C for 2 days, then placed unopened at varying storage temperatures. Virus activity was shown by mouse inoculation. All determinations were run in triplicate, and in no case was there appreciable variation in the three determinations. Table III shows the results obtained.

It is evident from Table III that the adapted influenza A virus survived under the various storage temperatures better than did the unadapted control virus.

A subculture of the 80th passage in tissue culture medium of the PR8 strain undergoing heat adaptation at 50°C for 60 min inter-transfer heat treatments was made in the chorioallantoic sac of 12-day embryonated

TABLE II.
Survival of Heat-adapted PR8 Strain of Influenza Virus in Tissue Culture Medium After Single Heat Treatment.

Temperature °C	Time min	Survival
60*	5	+
	7	0
55	15	+
	30	+
	45	±*
	60	0
50	60	+
	120	+
	180	+

* Occasionally no virus survived.

TABLE III.
Survival of Unadapted and Heat-adapted PR8 Influenza Virus in Tissue Culture Medium After Storage.

Temp of storage °C	Survival unadapted days	Survival time-adapted days
25.5	28	49
37	18	22
41	8	14

⁷ Henle, Werner, and Henle, Gertrude, *Am. J. Med. Sci.*, 1944, **207**, 705.

⁸ Henle, Werner, and Henle, Gertrude, *Am. J. Med. Sci.*, 1944, **207**, 717.

eggs. Here the virus withstood the same 50°C heat treatment as in tissue culture, but passage with higher temperature heat shock was not possible.

In order to test further the effect of heat on the PR8 strain a change in procedure was made. Two passages instead of one were allowed between heat shock treatment. The virus-containing allantoic fluid was diluted with equal parts of broth before heat treatment, and the heated virus was adsorbed with a heavy suspension of sterile embryonic chick red blood cells after heating. The red cell-virus mixture was allowed to stand at room temperature until the hemagglutination was marked, and was then used for inoculation of the allantoic sac. It was thought that the inactivated virus would not be as readily eluted as was the active virus, and that this procedure would thereby minimize the inhibiting effect of inactive virus. Heat shock at 56°C for 15 min periods was used for 4 heat treatments, 56°C for 20 min periods for 10 more heat treatments, and finally the virus was able to withstand heat shock of 56°C for 30 min intervals, but was lost on the 20th heating. In several attempts to carry the virus for a greater number of heat shock treatments, the virus died out at a similar stage.

A control of unadapted PR8 strain was subjected to the same procedure, excepting for heat shock. This control and the adapted strain were tested for their ability to withstand single heat shock, with results as shown in Table IV.

Presence of virus was determined by the Rickard⁹ modification of Hirst's^{5,10} hemagglutination technic. Titrations of the un-

TABLE IV.
Survival of Unadapted and Heat-adapted PR8 Strain of Influenza Virus in Chorioallantoic Fluid After Single Heat Treatment.

Temp °C	Time min	Survival-unadapted	Survival-adapted
60	5	0	0
56	10	+	+
	15	±*	+
	20	0	+
	30	0	+
	45	0	±
	60	0	0

* Occasionally no virus survived.

TABLE V.
Survival of IC Strain of Influenza Virus in Chorioallantoic Fluid After Single Heat Treatment.

Temperature °C	Time min	Survival
60	5	+
	7	0
56	15	+
	20	+
	30	+
	45	0
	60	0

adapted PR8 strain control usually gave endpoints of 1-200. Titrations of the adapted virus showed only slight, if any, agglutination in the first passages following heat shock treatment, and usually gave endpoints of 1-200 in the second passages. This was probably due to the inhibitive effect of the inactivated virus.

In order to determine whether changes in specificity had occurred during the adaptation to heat, the adapted virus was set up with antiserum to the unadapted control PR8 virus; neutralization occurred.

The heat-adapted and unadapted strains of virus were mixed with a culture of *Eberthella typhosa*, and heated at 56°C for 30 min. The typhoid bacillus and the unadapted virus did not survive this treatment. However, the adapted strain was not inactivated.

A more rapid method of adaptation was attempted using PR8 strain grown in the chorioallantoic sac. Heatings at 56°C for periods increasing by 5 min intervals from 5 to 30 min were used; each heating was followed by chick embryonic red cell adsorption and 2 transfers in the allantoic sac, and each period of time was used twice. The virus became able to survive the two heat treatments at 56°C for 25 but not 30 min periods. This was indication of an increase in ability to withstand heat as compared with the unadapted strain, but was under the limit attained by the slower procedure.

A recently isolated strain of influenza A virus, the IC strain, was included in the study. Preliminary tests of the ability of the IC virus to withstand heat were carried out with virus

⁹ Rickard, E. R., Thigpen, Minnie, and Crowley, James H., *J. Immunol.*, 1944, **49**, 263.

¹⁰ Hirst, George K., *J. Exp. Med.*, 1942, **75**, 49.

grown in the chorioallantoic sac of 12-day embryonated eggs. The results are shown in Table V.

Comparison of the initial heat resistance of the PR8 and IC virus strains in Tables IV and V shows a greater ability of the IC virus to withstand heat.

When the IC strain was heated for second treatments at 56°C for 30 min periods, active virus could not be shown in culture following the second heat treatment.

The IC strain was subjected to the more rapid method of adaptation. Heatings at 56°C for periods increasing by 5 min intervals from 10 to 30 min were used; each heating was followed by chick embryonic red cell adsorption and 2 transfers in the allantoic sac, and each period of time was used 4 times. The IC strain became able to survive two heat treatments at 56°C for 30 min periods, but was lost repeatedly on additional heatings. Using the Salk¹¹ modification of Hirst's hemagglutination technic, which gives agglutinations at greater virus dilutions than the Rickard technic, endpoints were obtained at dilutions of virus of 1-480 in the first passage after heating, and 1-4000 in the second passage. The IC virus strain controls were treated in the same manner, excepting for heat shock, and they also showed titers of 1-4000. Single

heat shock tests of the adapted virus surviving the first heat treatment at 56°C for 30 min gave the results seen in Table VI.

It was evident that this IC strain of virus, which normally had a higher resistance to heat than the PR8 strain, could also be adapted to withstand heat treatment which would normally destroy the unadapted IC virus.

Discussion. Growth of influenza A virus was shown to take place at an elevated temperature (41°C), but no evidence of adaptation of the virus to heat by cultivation at that temperature could be demonstrated.

Influenza virus could be adapted to heat by heat shock treatment between transfers. The procedure which was developed minimizes the antagonistic action of inactivated virus on the growth of active virus by allowing: (1) at least two transfers between each heat treatment, (2) some dilution of the virus-containing fluids, and (3) adsorption of virus with fowl red blood cells. A technic such as this was particularly necessary when temperatures and duration of treatment approximated very closely the inactivation conditions for unadapted virus. The presence of heat-inactivated virus exerted a marked inhibitory effect on the propagation of active virus, and repeatedly exerted a cumulative effect so that successive transfers under a particular condition became impossible. It may be possible to increase the ability of the influenza virus to withstand even greater temperatures for longer periods of time by long-continued transfers with very gradually increasing heat shock.

Conclusions. 1. Two strains of influenza A virus were trained to withstand temperature conditions which were normally lethal. 2. Influenza A virus could not be adapted to heat by growth at elevated temperatures.

TABLE VI.
Survival of Heat-adapted IC Strain of Influenza
Virus in Chorioallantoic Fluid After Single
Heat Treatment.

Temperature °C	Time min	Survival
60	5	+
	7	0
56	15	+
	20	+
	30	+
	45	+
	60	0

¹¹ Salk, Jonas E., *J. Immunol.*, 1944, **49**, 87.

Observations on Mammary Growth in Parabiotic Rats.*

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Although the use of parabiotically joined animals for investigations of the hormonal control of mammary growth has been limited,^{1,2} this method seemed to offer an approach to the problem of the nature of the anterior pituitary mammotropic substance and of the mechanism of interaction of hypophyseal and sex hormones on the mammary glands. The preliminary results of such a study on the mammary gland of the rat are here presented.

Methods. Littermate rats of the same or different sexes were joined parabiotically when 23 to 46 days old. In most cases one or both members of a pair were castrated a few days previously. A modification of the method of parabiosis described by Bunster and Meyer³ was employed. Coelomic cavities remained separate. After one to 3 weeks a castrated member of each pair was hypophysectomized. In most cases 10 γ of estradiol dipropionate[†] were injected daily for 10 days into one member of the pair, usually the hypophysectomized one. Single hypophysectomized rats of comparable age served as controls. At autopsy the inguinal mammary glands were prepared as whole mounts, and thyroids and adrenals were weighed and fixed. Completeness of hypophysectomy was verified by histological examination of the sellae turcicae.

Results. Mammary glands of single hypophysectomized control rats consisted only of slender, naked ducts. In the mammary glands of 21 of 24 hypophysectomized rats living in parabiosis with rats with hypophysis intact new growth occurred or regression was pre-

TABLE I.
Mammary Responses in Estrogen-Treated Hypophysectomized Rats in Parabiosis with Rats with Intact Hypophyses.

Group	Combination of parabionts		Mammary responses in hypophysectomized rat ¹			
	Hypophysectomized rat	Partner	+++	++	+	o
I	♂ castrate	♀	5	4	2	0
II	♂ "	♀ castrate	0	0	0	1
III	♂ "	♂ "	0	0	5	0
IV	♀ 2	♀	0	0	2	1
V	♀ castrate	♀	1	0	0	0
VI	♀ "	♀ castrate	1	0	1	1

¹ Mammary responses have been evaluated as follows: +++, greatest growth; ++, moderate growth; +, slight growth or prevention of regressive changes; and o, no prevention of regression.

² No estrogen injected.

vented (Table I). This response was evidenced by growth of ducts and end buds, thickening of the ducts, and the presence of numerous lateral buds. The cases of greatest growth were all confined to pairs wherein the animal with pituitary intact was a female or female castrate. Weights of adrenals and to a lesser extent of the thyroids of hypophysectomized parabionts exceeded those of single hypophysectomized rats of similar size although they did not equal those of normal animals.

Discussion and conclusions. Since estrogen does not stimulate mammary growth in hypophysectomized rats of the age and size of those used in these experiments,^{4,5,6} it may be concluded that some pituitary gland substance must have crossed from the partner with hypophysis intact to the hypophysectomized partner and there made possible the mammary growth observed. In this respect the hormone which possesses mammogenic potency is shown

* Aided by a grant from the Sackett Fund of the Trustee-Faculty Committee on Research.

¹ Matsuyama, R., *Frankf. Z. Path.*, 1921, **25**, 436.

² Hill, R. T., *J. Exp. Zool.*, 1932, **63**, 203.

³ Bunster, E., and Meyer, R. K., *Anat. Rec.*, 1933, **57**, 339.

[†] The hormone was supplied by Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J.

⁴ Selye, H., Collip, J. B., and Thomson, D. L., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1377.

⁵ Reece, R. P., Turner, C. W., and Hill, R. T., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 204.

⁶ Leonard, S. L., *Endocrinology*, 1943, **32**, 229.

to resemble other known pituitary hormones of protein nature. Such passage between parabiotic animals of gonadotropic, adrenotropic and growth hormones has been previously described^{7,8,9} and was also observed in the present experiments. The steroid hormone estrogen, on the contrary, failed to be transferred to the partner.⁸ If the pituitary mammotropic substance has a lipid character

as is reported by Lewis and Turner¹⁰ it is perhaps surprising that its behavior in parabiosis seems to link it more with the protein hormones than with the chemically more similar estrogen.

Since the pituitary gland of one rat was serving both members of a pair the amount of mammotropic factor which crossed to the hypophysectomized partner must have been small; yet this quantity was sufficient to alter the mammary response to estrogen from an unqualified negative to a definite positive. Furthermore the fact that the greatest mammary growth occurred where a female was furnishing the mammotropic hormone to the hypophysectomized parabiont suggests a sex difference in production and release of this hormone.

⁷ Møller-Christensen, E., *Acta Path. et Microbiol. Scand.*, 1933, **10**, 296.

⁸ Van Dyke, H. B., *The Physiology and Pharmacology of the Pituitary Body*, University of Chicago Press, 1936, 1939.

⁹ Greep, R. O., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 214.

¹⁰ Lewis, A. A., and Turner, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 435.

14939

Cell Count, Rate of Flow, and Protein Content of Cervical Lymph in the Rat.*

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Previous studies^{1,2} have shown that it is possible to collect thoracic duct lymph from the anaesthetized rat in amounts sufficient to carry out cell counts and chemical analyses. It was noted in these studies that there was an evident flow of lymph from the cervical lymphatic trunk in the preparations employed. Since spontaneous flow of lymph from the periphery (head and neck or extremities) of quiescent animals is unusual,³ it is thought noteworthy that there is, in the anaesthetized

rat, a steady flow of lymph from the main lymphatic trunks draining the head and neck. The present report describes a method of cannulation of the cervical lymphatic trunk, and presents data on the rate of flow, white cell count, and protein content of the lymph obtained. This data is compared with further measurements of the same type for thoracic duct lymph.

Technic of collection of cervical lymph. Adult female rats of the Long-Evans strain, unfasted, weighing between 250-300 g, were anaesthetized by intraperitoneal injection of a 1% solution of sodium pentobarbital. A midline incision was carried through skin and subcutaneous tissue from the symphysis menti to the sternal angle. The incision was carried sufficiently deep to expose the sternohyoid muscles. All superficial neck tissues were retracted laterally. The manubrium sterni was split lengthwise in the midline, using bone

* Assisted by grants from the Research Board of the University of California and the Rockefeller Foundation of New York City.

¹ Reinhardt, W. O., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 123.

² Reinhardt, W. O., and Li, C. H., *Science*, 1945, **101**, 360.

³ Drinker, C. K., and Yoffey, J. M., *Lymphatics, Lymph and Lymphoid Tissue*. Harvard University Press, Cambridge, Mass., 1941.

TABLE I.

Animal No.	Duration of lymph collection		Wt of lymph (mg)	Lymph flow/hr (mg)	WBC/cmm	Protein* (g %)
	hr	min				
41	0	48	38.5	48.1		
42	2	0	96.0	48.0	4,800	
43	0	35	13.5	23.2	9,050	
44	6	20	196.3	31.0	2,400	4.12
45	5	50	300.1	51.4	4,250	2.46
46	4	10	98.9	23.7		3.31
47	7	0	177.9	25.4	5,200	3.44
49	6	5	204.8	34.0	1,525	3.44
50	1	45	112.2	64.2	15,050	2.87
51	9	55	418.9	42.3	8,500	2.56
52	8	0	218.5	27.2	2,375	3.00
53	8	40	375.3	42.3	17,500	2.44
Avg				38.5	7,065	3.07

* The total protein ($N \times 6.25$) represents g/100 g of lymph (not corrected for N P N).

forceps. The two halves of the manubrium were retracted laterally by means of hemostats. The attachments of the sternohyoid muscle to the manubrium were dissected away bluntly, exposing the carotid arteries, vagus nerve and internal jugular veins on each side of the neck. Lateral to and in the same layers of deep cervical fascia as the structures of the carotid sheath, course the cervical lymphatic ducts from the head and neck to empty into the venous system; on the left, via the common lymph sac by union with the thoracic duct, and on the right, to empty into the venous system at the junction of internal jugular and subclavian veins. The lymph vessels were isolated (under the binocular microscope) by blunt dissection at the site of their drainage into the venous system and were ligated by single silk ligatures to prevent reflux of fluid. Ligation of the lymph trunks produced immediate distention allowing one to choose for cannulation an area in the vessel wall free from the rather extensive plexus of vasa vasorum. The point of a 27 G hypodermic needle was thrust through the vessel wall, it being possible to distinguish the layers of the vessel wall grossly. When the needle passed through the intimal layer, a free flow of lymph ensued. A fine glass cannula with tip of the same size as the needle was then threaded into the vessel and left *in situ*. It was not necessary to tie the vessel on the cannula; the muscular wall of the lymphatic sufficed to maintain contact with the cannula. Crystal-clear lymph flowed readily into the cannula and, indeed, against

a pressure of 1-2 cm of water, since the end of the cannula rested on the thoracic cage. It was possible to cannulate both cervical lymph trunks in the same animal. Lymph was collected over a variable number of hours, expelled into weighing bottles, weighed, thoroughly mixed for white blood cell sampling (duplicate), and analyzed for total nitrogen content by a micro-Kjeldahl method.

Results. Table I presents the pertinent data gathered on cervical lymph in the anaesthetized rat.

The data on cervical lymph may be compared in Table II with similar data for thoracic duct lymph, obtained under identical conditions in another series of rats of the same age and weight.

It will be noted that the absolute lymphocyte count of cervical duct lymph is about $\frac{1}{3}$ that of the thoracic duct, and that the rate of flow is about $\frac{1}{10}$ as great.

It is evident that the motionless anaesthetized rat exhibits a free flow of cervical duct lymph. This is in distinction to other commonly used laboratory mammals.³ For this reason, the rat may be useful in the study of absorption of various particulate materials, protein solutions, or other foreign material, from organs or tissues of the head and neck, without the necessity of resorting to artificial methods of producing passive motion or massage to promote lymph flow.

Preliminary electrophoretic analyses of blood plasma and cervical lymph (pooled specimen) indicate that there is no essential

TABLE II.

	Thoracic duct lymph		Cervical duct lymph	
White cells/cmm	21,250 \pm 1,580*	(37)†	7,065 \pm 1,725,	(10)
Lymph flow/hr	0.39 \pm 0.03 cc	(27)	38.5 \pm 3.75 mg	(12)
Protein conc. (whole lymph)	4.18 \pm 0.11 (g %)‡	(19)	3.07 \pm 0.18 (g/100 g)§	(9)

* Mean \pm S.E.

† Figures in parentheses indicate number of animals.

‡ Copper sulfate gravity method. (Avg specific gravity of 19 specimens was 1.0187 \pm 0.00014)§ Micro-Kjeldahl. (N \times 6.25) (not corrected for N P N)

difference in the albumin-globulin ratio, but that there is a definitely higher content of gamma globulin in the cervical lymph as compared with blood plasma.

Summary. The quiescent anaesthetized rat exhibits a free flow of lymph from the cervical lymphatic trunks. A method of collecting

cervical lymph in the rat is described. The average rate of flow is approximately 40 mg/hr, the lymphocyte count averages 7,065/cmm, and the average protein content is 3.07 g %. These figures are compared with similar data for thoracic duct lymph.

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Comparison of the Toxicity of Antiseptics for Embryonic Tissue and Bacteria.

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(Introduced by A. C. de Graff.)

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This report describes a procedure for the determination of the toxicity index of antiseptics which appears to have certain theoretical and practical advantages over those previously presented.

The evaluation of antiseptics has long been a difficult problem, largely because of the many types of chemical compounds which have been found useful in this field. To date, no entirely successful procedure has been devised for comparing these drugs except when tests are made of closely related compounds. The use of a toxicity index (the ratio of the dilution of an antiseptic which will produce some readily determined toxic effect on tissue to that which will kill bacteria tested) permits interclass comparisons to some extent. The reliability of the results can be enhanced further by inclusion of a suitable reference standard to control shifts in tissue or bacterial susceptibility. In addition it is generally recognized that since the toxicity of an antiseptic is frequently

modified by the presence of organic matter, the tissue or bacteria should be implanted in the medium before contact with the antiseptic is permitted. In a recent paper Salle and co-workers¹⁰ have described a method which takes into account all of the foregoing considerations. They suspended chick heart fragments in serum and embryonic extract, added antiseptic, and then, after 10 min exposure, cultured the tissue in Carrel flasks. In a corresponding series of experiments, bacteria were exposed for 10 min followed by subculture in beef broth.

Salle's work and that of other investigators¹⁻¹⁰ in this field has been based on either

¹ Lambert, R. A., *J. Exp. Med.*, 1916, **24**, 683.

² *ibid.*, *J. Am. Med. Assn.*, 1916, **67**, 1300.

³ Lambert, R. A. and Meyer, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1926, **23**, 429.

⁴ German, W. J., *Arch. Surg.*, 1929, **18**, 1920.

⁵ Buchsbaum, R., and Bloom, W., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 1060.

TABLE I.
Toxicity of Phenol for Embryonic Tissue and Bacteria.

Dilution	Fraction of heart fragments showing growth 48 hr after implantation			Dilution	Growth of <i>Staphylococcus aureus</i>		
	1st trial	2nd trial	3rd trial		1st trial	2nd trial	3rd trial
1:535	0/10	0/10	0/10	1:205	—	—	—
1:666	0/10	0/10	0/10	1:256	—	—	—
1:840	10/10	7/10	10/10	1:321	+	+	+
1:1042	10/10	10/10	10/10	1:400	+	+	+
1:1315		10/10	10/10				

TABLE II.
Toxicity of Antiseptics Towards Tissue Cells and Bacteria and Derived Toxicity Index.

Antiseptic	Dilution inhibiting growth of approximately 50% of fragments (A)	Dilution lethal to <i>Staphylococcus aureus</i> (B)	Toxicity index (Ratio A/B)
Strong solution of Iodine, USP XII	1:1315	1:4570	0.29
Azochloramide	1:65,000	1:101,700	0.64
Phenol	1:782	1:288	2.7
Zephiran	1:73,000	1:21,200	3.4
Merthiolate	1:202,000	1:52,000	3.9
Saponated solution of Cresol, USP XII	1:1163	1:285	4.1

the Carrel flask or the hanging drop method. It was found that neither of these procedures was convenient for a quantitative method. Both methods depend upon passive diffusion alone as the means of exposing the biological material to the antiseptic. In the case of the hanging drop technic accuracy in measurement of media is difficult.⁵

Moreover, the use of a 10 min exposure period¹⁰ would seem to be an arbitrary condition imposed upon the experiments. This criticism is justified since not all antiseptics have the same wetting powers and it is impossible to plant pieces of tissue of exactly the same size and shape.

In the work reported below, Gey's method of cultivating tissue in roller tubes^{11,12} has been adapted to the study of antiseptics. The

continual movement imparted to cells and media seems to provide a closer approach to *in vivo* conditions. Luxuriant growth can be obtained and accuracy in measurement of media and antiseptic solutions is possible.

Procedure. The most convenient tube was found to be a vial 100 mm long by 25 mm wide with a neck 15 mm in diameter. It was sealed with a paraffined cork. With the aid of a pipette about one cc of chicken blood plasma was transferred to the tube and the tube gently rotated until the plasma was spread uniformly over the inner surface. The excess was removed with a pipette after allowing the tube to stand upright for a few minutes. Ten to 15 such tubes were prepared by this process called "lining" by Gey.¹¹ The tissue cells or the bacteria were then introduced into separate series of tubes. If tissue was being studied ten fragments of heart ventricles 0.5 to 1 cu mm in volume from 9-day embryonic chicks were set in the plasma lining. Where bacteria were being studied one 4 mm loopful of a 24-hr beef heart infusion culture of *Staphylococcus aureus* was streaked up the sides of the lining. In either case the tubes were stoppered and placed horizontally in a motor-driven drum rotating at a speed of 10 revolutions per hour in an incubator at 38.5°C and the plasma was allowed to clot. Each tube

⁶ Salle, A. J., and Lazarus, A. S., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 665, 937, 1057, 1119, 1481.

⁷ *ibid.*, 1935, **33**, 8, 393.

⁸ *ibid.*, 1936, **34**, 371.

⁹ Salle, A. J., McOmie, W. A., Shechmeister, I. L., *J. Bacteriology*, 1937, **34**, 267.

¹⁰ Salle, A. J., McOmie, W. A., Shechmeister, I. L., and Foord, D. C., *Proc. Soc. Exp. Biol. and Med.*, 1938, **37**, 694.

¹¹ Gey, G. O., *Am. J. Cancer*, 1933, **17**, 752.

¹² Gey, G. O., and Gey, M. K., *ibid.*, 1936, **27**, 45.

was then removed momentarily from the incubator and 0.75 cc of embryonic extract was added followed by 0.75 cc of one of a series of antiseptic dilutions and the tube was agitated gently so as to assure complete mixing. In this way the gutter of embryonic extract and antiseptic extended the length of the lined surface and bathed the tissue cells approximately once every 6 min. All tubes were left in the incubator for a period of 48 hr. In the case of a tube containing tissue fragments, growth or no growth of each of the ten pieces of tissue was checked with the low power binocular microscope and recorded at the end of this period. In the case of a tube containing bacteria a loopful of the fluid material was transferred to a tube containing 10 cc of beef broth and incubated for an additional 24 hr. At the end of this time each tube was examined for the presence of staphylococci. Gross evidence, such as turbidity or lack of it, was supplemented by microscopic examination of stained smears. Routinely the beef broth tubes were saved for an additional 2 or 3 days as a check on possible bacteriostasis.

The stock solutions of antiseptics were as follows: saponated solution of cresol USP XII, strong solution of iodine USP XII, zephiran aqueous concentrate 10%, 1% merthiolate solution prepared from powder, 1% azochloramide solution prepared from the commercial tablets and 5% phenol solution prepared from the pure crystals. The dilutions were made from the above stock solutions with Tyrode solution containing 10% embryonic extract. The embryonic extract presumably acts as a protective colloid and prevents the precipitation which would otherwise take place in the case of cresol.[†]

[†] The saponated solution of cresol causes a flocculent precipitate of calcium and magnesium soaps when added to Tyrode solution. In the presence of embryonic extract these insoluble soaps remain finely divided and do not interfere mechanically with the procedure. The soap may play a considerable part in the antiseptic action of the phenolic antiseptics, especially when they are used in the presence of serum or exudate. Soap is, of course, necessary to maintain the phenol homologues dispersed in water.

The first experiments were of necessity orienting or range finding. In these the antiseptic dilutions were varied by a factor of 2 as 1:100, 1:200, and 1:400. After determining the range of toxicity further dilutions were made which differed by a factor of 1.25. In other words, each successive dilution differed by 25% from the preceding. It was possible to differentiate with ease tissue in which no appreciable migration of fibroblasts had taken place, from tissue showing unmistakable growth along the periphery. Border line cases were so rare as to permit scoring a group of fragments in terms of an all or none response. In the case of the tissue growth experiments six dilutions were usually set up for each drug so that those causing zero and 100% growth inhibition fell as nearly as possible in the middle of the group of 6. The median toxic dilution, that is, the dilution inhibiting growth in 50% of the heart fragments, was estimated as the midpoint between the zero and 100% values. Solutions for the study of bacterial growth were set up in like manner and the bactericidal dilution was taken as the average of a dilution which fails to kill and another dilution 25% smaller which is lethal. Phenol was used as a standard of comparison and a series of dilutions were tested with every group of experiments either with tissue or bacteria.

Results. The reproducibility of the results may be judged from Table I. It is evident that the data are entirely consistent and no shifts occurred in the susceptibility of tissue or organisms. In most instances the slope of the curve relating dilution to percent inhibition for tissue was quite steep so that by using a smaller factor between dilutions and larger groups of heart fragments, a median inhibiting dilution with its standard error might be readily determined.

In Table II are given the dilutions of the antiseptics toxic to tissue and lethal to bacteria and the toxicity indices derived from them. The toxicity indices have been arranged in ascending order; the larger the value the less favorable is the index. It will be evident that, only where the index is less than one, will the dilution of the antiseptic which is lethal to staphylococci be greater than the dilution which inhibits the growth of tissue.

The results are in agreement in order of magnitude with those of Salle⁶⁻¹⁰ and Lambert.¹⁻³ The phenolic compounds and merthiolate have indices considerably greater than one, whereas iodine, with an index of 0.29, is by far the best of all the drugs studied. Of the two drugs which have not been studied previously, zephiran has an index of the same order as that of the phenolic compounds, whereas azochloramide has an index approaching that of iodine.

The interpretation of these results in terms of various clinical applications must be made with caution. The toxicity indices have been determined here under conditions similar to those which obtain when an antiseptic is

applied to denuded tissue in the presence of serous exudate. When an antiseptic is applied to intact skin or mucous membrane the index will probably be different, and there is no reason to expect parallelism with the values reported above.

Summary. A method has been described for quantitative comparison under identical physiological conditions of the toxicity of antiseptics for tissue and bacteria. The technic is simple and the results are reproducible. The toxicity indices were found to be as follows: iodine, 0.29; azochloramide, 0.64; phenol, 2.7; zephiran, 3.4; merthiolate, 3.9; saponated solution of cresol USP XII, 4.1.

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Occurrence of Stainable Lipoid Material in Renal Epithelium of Animals Falling in Different Age Segments.*

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In previous publications¹⁻³ observations have been recorded of variations in the occurrence of stainable lipid material in different segments of the renal nephron. The factor of age associated with these variations has been noted. The present study permits an extension and amplification of certain of these observations.

The first series of experiments represented by 110 dogs includes animals varying in age from 6 weeks to 18 years and 3 months. On the basis of age these animals have been divided into 3 groups. Group I, composed of 31 dogs between the ages of 6 weeks and 1 year; Group II, 52 dogs varying in age from

1 to 7 years; and Group III, 27 animals between 8 and 18 years and 3 months of age. Such a grouping permits observations of a cytological order that concern themselves with the tissues of puppies and young animals, Group I; adult animals, Group II; and older or definitely senile animals, Group III.

The dogs of the first series of experiments were not given any specialized diet but were allowed feedings once a day of scrap material from a large dining hall which usually contained scraps of meat, pieces of bone, bread and fats in the form of butter and tissue fat. Neither the food nor water intake was restricted. On 2 occasions, before the commencement of any experimental procedure, the blood and the urine of the animals were studied in order to eliminate the existence of a naturally acquired nephritis or some other form of renal injury. With the exception of 6 of the oldest dogs, in Group III, the urine was normal, the elimination of phenosulphonaphthalein in a 2-hr period was within normal percentage limitations, and there was no re-

* Aided by a grant from the Josiah Macy, Jr. Foundation.

¹ MacNider, Wm. deB., *J. Pharm. and Exp. Therap.*, 1921, **17**, 289.

² MacNider, Wm. deB., *PROC. SOC. EXP. BIOL. AND MED.*, 1922, **19**, 222.

³ MacNider, Wm. deB., *Problems of Ageing: Biological and Medical Aspects*, Williams & Wilkins Co., Baltimore, 1939.

tention of non-protein nitrogen or creatinine. In the 6 old animals that gave evidence of renal impairment the urine contained a heavy trace of albumin and showed the presence of broad, hyaline and finely granular casts. Fatty casts were not present. The 2-hr output of phenolsulphenonephthalein by these 6 old animals varied from 21 to 43%. The non-protein nitrogen of the blood was between 61 and 82.7 mg and creatinine between 2.1 and 3.8 mg per 100 cc.

The experimental procedure consisted in the removal from the kidney of a wedge-shaped piece of tissue without the use of an anesthetic of the methane series. The use of such an anesthetic body even for a very short period eliminates the validity of any deduction concerning the amount of lipid material occurring naturally in such tissue.⁴ For the operative interference procaine was used supplemented at intervals by nitrous oxide-oxygen inhalations. Renal tissue so obtained was cut into two portions, one piece of which was immediately frozen for sectioning while the remaining tissue was fixed in 10% formalin for future study. The sections were stained for lipid material by Herxheimer's Scharlach R. method. A study of such sections from the 3 groups of animals indicates the following differences in distribution and in amount of lipid material that can be demonstrated in the renal nephron. In Group I (puppies and young dogs), such material has been observed in the proximal convoluted segment of the nephron in only 4 of the older animals of this young group. In this location such material appears as fine dust-like granules of a more brick-red than orange-red hue, the latter being the usual order of color determination when such material is present in tissue in any considerable amount. Such granules are located on the lumen side of the cells of this segment of the nephron. They have not been observed in the cytoplasm immediately surrounding the nucleus of such cells. In the remaining animals of this young group, 32 in number, there has been no microchemically detectable lipid material in the cells of this segment of the nephron. In this Group I animals as well as

in the adult and in the older and senile animals, stainable lipid material occurs as a normal constituent for both the descending and ascending limbs of Henle's loop. Such material in these locations exists as coarse granules of a brick-red color. The incidence of the presence of this material in the loops of Henle becomes more marked as the animals advance in age. In the old and very old animals, with the exception of the 6 animals in Group III with evidence of renal damage, there is a striking difference in the amount and location of stainable lipid material appearing in the renal nephron. There is associated with the ageing of the animals and not with a state of renal disease the constant appearance of stainable lipid material in the cells of the proximal convoluted segment of the nephron. This observation is in marked contrast to the absence of such material in the same segment of the nephron in most of the puppies and young adult animals of Group I. In the adult, aged and senile dogs of Group II and III this material makes its appearance in the convoluted tubule cells as definite droplets or fused masses of an orange-red color which not infrequently obscures the nucleus of such cells. In general, it would appear that increments in such material first take place between the nuclei and the free lumen border of the cells.

Reference has been made to 6 animals of the old and senile group (Group III) that gave evidence both in the blood and urine of the existence of some order of chronic renal injury. Frozen sections from such tissue stained with Scharlach R, as well as sections stained with hematoxylin and eosin, have shown these dogs to have an advanced chronic glomerular nephritis with an associated repair process to the epithelium of the proximal segment of the nephron which has resulted in the formation of a changed type of cell, abnormal for this segment of the nephron. These cells, or if cell masses show poor cell differentiation, areas which are syncytial in configuration, the cells are formed of flattened, evenly and deeply staining cytoplasm, containing at irregular intervals prominent and intensely staining nuclei. This epithelial repair process has resulted in the formation of an embryonic order of tissue to replace the cuboidal, highly specialized cell

⁴ MacNider, Wm. deB., *J. Pharm. and Exp. Therap.*, 1920, **15**, 249.

units normal for this segment of the nephron. A study of sections of this tissue stained with Scharlach R has demonstrated the occurrence of stainable lipid material in the cells of the loops of Henle, which material is comparable in amount to that found in the remaining old and senile dogs of this group (Group III) which failed to give evidence of a renal injury. Other than the glomerular injury in the 6 nephritic animals of the group, and the changes in the configuration of the epithelial structure, the observation of importance is that stainable lipid material cannot be demonstrated in this embryonic order of replacement epithelium, while in animals of the same advanced age group in which no injury has existed to provoke an atypical repair process in the convoluted tubule segment the cells of this portion of the nephron are heavily loaded with stainable lipid.

A similar order of change in epithelial type has been observed for the liver in certain senile animals⁵ associated with a reduction in certain manifestations of hepatic function and furthermore in other animals⁶ not senile in which, following a severe injury from uranium nitrate, the liver underwent a process of atypical epithelial repair comparable to that described for the kidney in the present series of animals. With this order of repair for the liver, the cells and syncytial structure effecting the repair fail to show the presence of stainable lipid material. This type of epithelium is resistant to subsequent intoxications by uranium nitrate and is also resistant to chloroform.

The 110 dogs which have been the subject of this discussion were not on a constant diet of known composition. Variations, if persistent, in the chemical nature of such material might with some readiness influence the amount of stainable lipid material in the tissues of the animals. In order to minimize, if not eliminate, this factor 38 dogs between the ages of 4 months and 12 1/6 years have been maintained over periods of 3 months on

Purina Dog Chow. No limit was placed on the amount of such material consumed. The water intake was not restricted. Studies of the blood and urine of this group of animals, as has been outlined for the dogs of the previous series, indicated the animals were free from renal injury. Kidney tissue was obtained from these animals by the usual technic and frozen sections stained by the previously described method. The distribution and apparent amount of stainable lipid material in the cells of the renal nephron of these animals on a constant diet of known composition have been similar to those for the first and larger group of animals in which the diet was not controlled. Puppies of 4 months and the younger animals up to 1 year of age failed to show the presence of stainable lipid in the specialized cells of the proximal segment of the nephron. Such material was present in the cells of the loops of Henle. As the animals advanced in their age segments such stainable material made its appearance as small and larger droplets and as fused masses in the cells of the proximal convoluted segment.

Conclusions. 1. Microchemically demonstrable lipid material is not present in the epithelium of the cells of the proximal convolution of the nephron in puppies varying in age from 6 weeks to 4 months. Young adult animals under 1 year of age have rarely shown such material as fine dust-like granules. 2. As the dog ages, stainable lipid makes its appearance in the cells of the proximal convoluted segment and increases in amount in this location as the animal advances in years. 3. In nephritic senile animals that have repaired the epithelial injury in the proximal segment by the formation of an atypical, flattened order of epithelial structure stainable lipid material cannot be demonstrated by the use of a similar technic which does demonstrate its presence in cells of a normal order in this location. 4. Such variations in the occurrence of stainable lipid material are not due to qualitative variations in the food intake. 5. Intracellular chemical manipulations would appear to become so modified by the factor of age as to influence the amount and distribution of stainable lipid material in the cells of the proximal convolution of the nephron.

⁵ MacNider, Wm. deB., *J. Pharm. and Exp. Therap.*, 1936, **56**, 383.

⁶ MacNider, Wm. deB., *J. Pharm. and Exp. Therap.*, 1936, **56**, 373.

Inhibition of the α and β Isoagglutinins in Anti-RH Serums.

MERCEDES VICENTE DE TORREGROSA. (Introduced by P. Morales Otero.)

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Recently Oliver-González¹ described a polysaccharide isolated from the pig roundworm *Ascaris suum* which has the property of inhibiting the α and β agglutinins in human serums. Furthermore, Oliver-González and Montilla² reported that the action of the ascarid polysaccharide is limited to the α and β agglutinins with no effect on the anti-M, anti-N and cold agglutinins. These investigators therefore conclude that the polysaccharide may be used in the preparation of anti-Rh serums, where the α and β agglutinins need to be removed. Since the studies of Oliver-González and Montilla² on the treatment of anti-Rh serums with the ascarid polysaccharide, included only 2 human anti-Rh serums the work that follows was done to test the effect of this substance on a larger number of anti-Rh serums coming from various human sources.

Materials and Methods. The anti-Rh serums* used in this study were obtained from the following sources:

1. From a boy suffering from osteomyelitis. This patient was successfully given 8 blood transfusions before the appearance of atypical agglutinins.

2. From a case of subacute bacterial endocarditis. This patient developed demonstrable anti-Rh antibodies after 2 blood transfusions.

3. From a patient with bleeding peptic ulcer immunized through 6 blood transfusions.

- 4 and 5. From women who delivered infants that developed acute hemolytic anemia of the newborn.

The method used for the inhibition of isoagglutinins follows the one described by

Oliver-González and Montilla.² To small volumes of serums enough polysaccharide was added to make a 4% suspension. Such treated serums were incubated for 30 min at 37°C and left overnight in the icebox. The centrifuged and clear supernatants were then used as the test serums. With some serums further treatment with polysaccharide was found to be necessary.

The Rh positive and the Rh negative cells used in these experiments have been classified as such after testing them with anti-RH₀, anti-Rh' and anti-Rh'' serums. These test serums determine the 8 Rh types described recently by Wiener³ and which are designated as follows: Rh negative, Rh₁, Rh₂, Rh₁Rh₂, Rh₀, Rh', Rh'' and Rh'Rh''.

Agglutination tests were done by the test tube technic recommended by Levine⁴ that is more sensitive in detecting weak reactions such as those due to the Rh factor. One drop of anti-Rh serum and one drop of 2% red blood cell suspension in saline were mixed in a small test tube and incubated for one half hr at 37°C. The quality of the sediment was observed then centrifuged and by means of a mirror read macroscopically while shaking gently. A drop placed on a slide was also read microscopically for evidence of clumping. Tubes showing agglutination of the erythrocytes were considered positive.

Results and Summary. As may be seen in Table I, in the majority of serums the polysaccharide inhibited the α and β agglutinins when this was added to the serums to a concentration of 4%. The anti-Rh agglutinins remained unaltered. In serum No. 4, however, addition of polysaccharide to make a concentration of 12% was necessary in order to inhibit the β agglutinins. In serum No. 5 the β agglutinins could not be removed from the

¹ Oliver-González, J., *J. Infect. Dis.*, 1944, **74**, 81.

² Oliver-González, J., and Montilla E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 169.

* These test serums were obtained through the courtesy of Dr. Alexander S. Wiener of the Brooklyn Jewish Hospital, New York City, N. Y.

³ Wiener, A. S., *Science*, 1944, **99**, 532.

⁴ Levine, Ph., Katzin, E. M., and Burnham, L., *Proc. Soc. Exp. Biol. and Med.*, 1944, **45**, 346.

TABLE I.
Effect of Ascarid Polysaccharide on the α and β Isoagglutinins in Various Anti-Rh Human Serums.

Serum No.	Agglutinins in serum	Conc. of polysaccharide in serum %	Untreated serums							
			Group A		Erythrocytes tested				Group O	
			Rh+	Rh—	Group B Rh+	Group B Rh—	Group AB Rh+	Group AB Rh—	Group O Rh+	Group O Rh—
1	β		+	—	+	+	+	+	+	—
2	β		+	—	+	+	+	+	+	—
3	α and β		+	+	+	+	+	+	+	—
4	α " β		+	+	+	+	+	+	+	—
5	α " β		+	+	+	+	+	+	+	—
6	α		+	+	+	—	+	+	+	—
Serums treated with ascarid polysaccharide										
1	β	4	+	—	+	—	+	—	+	—
2	β	4	+	—	+	—	+	—	+	—
3	α and β	4	+	—	+	—	+	—	+	—
4	α " β	12	+	—	+	—	+	—	+	—
5	α " β	16	+	—	+	+	+	+	+	—
6	α	4	+	—	+	—	+	—	+	—

serum even after the addition of the polysaccharide to a concentration of 16%. Difficulty in removing the natural β isoagglutinins from some anti-Rh serums has been also encountered by Levine⁵ when the specific substances A and B were added to serums. Work is in progress to determine the relations that might exist between these non-absorbable natural isoanti-

bodies and those resulting from isoimmunization to the different Rh types.

The results of this study also confirm and extend the findings of Oliver-González and Montilla indicating that the polysaccharide from *Ascaris suum* may be used to inhibit the α and β isoagglutinins in human serums so that they can be used to detect Rh positive and Rh negative cells.

⁵ Levine, Ph., *Arch. Path.*, 1944, **37**, 83.

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Characteristics of Diphtheria Bacilli Found in Baltimore Since November, 1942.*

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In Baltimore from time to time during the past 22 years systematic examinations have been made of diphtheria in its clinical, epidemiological, immunological and bacteriological aspects. Reports have been published concern-

ing these various matters, including data on the distribution of diphtheria bacilli, fluctuations in ratio of virulent to avirulent strains, changes in carrier rate, the occurrence of various biological types such as the so-called gravis and mitis varieties, and miscellaneous facts relating to the natural history of diphtheria.^{1,2} It is the purpose of the present paper to present new data accruing from

* These studies were aided in part by a grant from the American Public Health Association.

† Of 169 virulent strains in this survey in which colony form was noted 92 formed minute colonies while 77 were large colony formers. Not all minute colony formers are minimus type organisms (Table II).

¹ Frobisher, M., Jr., *Am. J. Hyg.*, 1938, **28**, 13.

² Phair, J. J., and Smith, M. R., *Am. J. Hyg.*, 1942, **35**, 47.

TABLE I.
 Fermentation Reactions of Various *Corynebacteria*, Baltimore, 1942-1944.

Nature of reaction	Cultures	Dextrose*				Saccharose*			
		Fr. Au.	St. Au.	Fr. Fil.	St. Fil.	Fr. Au.	St. Au.	Fr. Fil.	St. Fil.
Typical	13	+	+	+	+	—	—	—	—
Dextrose irregular, saccharose neg.	30†	—±	—±	—±	—±	—	—	—	—
Saccharose pos.	4	+	+	+	+	+	+	+	+
<i>C. xerose</i>		+	+	+	+	—	—	—	—
<i>C. pseudodiph.</i>		—	—	—	—	—	—	—	—

* Fr. Au. = Fresh, autoclaved tubes; St. Au. = Stored, autoclaved tubes; Fr. Fil. = Freshly prepared with filtered sugar; St. Fil. = prepared with filtered sugar but stored in refrigerator before inoculation.

† The presence of +, —, and ± signs indicates that some strains fermented strongly but not in every tube, others failed to ferment, while others fermented slightly or slowly in one or more tubes.

similar studies carried on during the period from 1942 to the close of 1944.

Materials and Methods. The methods used throughout this work were the same as during the last 8 years in these laboratories and have been previously described.¹ From November 27, 1942 to June 23, 1944, 210 strains of bacteria morphologically typical of *Corynebacterium diphtheriae* were isolated from a total of about 750 throat cultures representing some 500 clinical cases, their contacts, and various healthy carriers. As in previous studies these bacteria were examined biologically either immediately on isolation or after varying periods of storage desiccated *in vacuo*. Virulence was tested by either the rabbit intradermal method of Fraser³ or by the chick method,⁴ or both. When both tests were used, no disagreements in result were observed. As a result of these tests 169 of the 210 strains were found to be virulent.[†] The present report deals principally with these virulent cultures. All of the cultures were also tested for 2 biochemical properties heretofore regarded as the *sine qua non* of identification as diphtheria bacilli, namely prompt, vigorous fermentation of dextrose and nonfermentation of saccharose. Most of the cultures were also examined for cultural properties characteristic of the gravis and mitis

 TABLE II.
 Dextrose Fermentation by Strains of *C. diphtheriae* Producing Minute Colonies.

Animal test	Dextrose fermented promptly	Dextrose fermented slowly, slightly or irregularly	Totals
Virulent	4	22	26
Avirulent	2	8	10
Totals	6	30	36

types. It has been pointed out previously⁵ that in these publications the terms gravis and mitis are used to indicate diphtheria bacilli having certain cultural characteristics and have no reference to virulence or clinical details.

Results. 1. Gravis and Mitis Types. Only 1 gravis-type culture was isolated during the entire survey. This was obtained during the convalescence of a patient who had yielded a mitis-like culture during his attack of diphtheria and whose sister, having diphtheria later on, also yielded a mitis-like strain on culturing. Thus the gravis-mitis situation in Baltimore seems at present to be not significantly different from what it was when similar surveys were made some 5 or 6 years ago.¹ That is, gravis type organisms are rare.

2. Atypical fermentations of dextrose and

 TABLE III.
 Dextrose Fermentation by Strains of *C. diphtheriae* Producing Large or "Ordinary" Size Colonies.

Animal test	Dextrose fermented promptly	Dextrose fermented slowly, slightly or irregularly	Totals
Virulent	12	3	15
Avirulent	4	2	6
Totals	16	5	21

³ American Public Health Association, *Diagnostic Procedures and Reagents*, Chapter on Diphtheria. New York, 1941.

⁴ Frobisher, M., Jr., Parsons, E. I., and Tung, T., *Am. J. Hyg.*, 1942, **35**, 381.

⁵ Frobisher, M., Jr., *Am. J. Pub. Health*, 1943, **33**, 1244.

saccharose. Of primary importance to the laboratory bacteriologist is the fact that, during the study, numerous strains of diphtheria bacilli were encountered which gave atypical reactions in the fermentation of dextrose or saccharose, *i.e.*, some fermented saccharose, some failed to ferment dextrose regularly.

Because of the importance of these observations with respect to the laboratory diagnosis of diphtheria it was deemed advisable to test several times the dextrose and saccharose fermentation of a number of these cultures taken at random, arranging the tests as a comparative experiment in which the carbohydrates were prepared in various ways, some conducive to hydrolysis of saccharose. The methods of preparation were as follows:

1. The sugars were added to infusion broth with indicator, tubed, autoclaved and inoculated as soon as cool.

2. Tubes of broth were similarly prepared but stored several months in the icebox.

3. Tubes of broth were similarly stored but the sugars were prepared in 10% aqueous solution, sterilized by filtration and added aseptically to the broth before storage.

4. Tubes were prepared with filtered sugar solutions as in 3 but were inoculated immediately after preparation.

All tubes contained brom cresol purple. The inocula consisted of 1 or 2 drops of 48-hr broth culture per tube. All tests were inoculated within a period of 2 hours and were incubated simultaneously. Observations were made on the 7th and 14th days after inoculation. There were few significant changes after 7 days.

Table I shows the results obtained with 47 virulent strains tested in the manner indicated. It is seen that 4 cultures regularly fermented both dextrose and saccharose. Without unduly enlarging the table it is not possible to convey accurately the irregularity in dextrose fermentation exhibited by many of the other cultures. Some strains fermented slightly or slowly in all tubes; some fermented strongly in one or two tubes and little or not at all in others. There was nothing to suggest that method of preparation of fermentation tubes, either of dextrose or saccharose, affected the result. It is evident that a single dextrose fermentation test does not serve to demon-

strate the full potentialities of diphtheria bacilli and that, in diagnostic laboratories, virulence tests had better be made with all morphologically suspicious organisms whether or not they give typical fermentation reactions.

Avirulent strains, morphologically indistinguishable from toxigenic *C. diphtheriae*, and having aberrant fermentative properties as described above, were encountered with the virulent ones but, since virulence is absent, differentiation of such strains from *C. xerose* and *C. hoffmanni* cannot be absolute, although fairly certain on morphological grounds.

3. *Small colony (minimus type) of C. diphtheriae*. A third observation made during these studies was the appearance of a variety of diphtheria bacilli characterized by the production of remarkably small colonies on either cystine-tellurite agar as regularly used for isolation, or on McLeod's tellurite agar used to bring out the distinctive properties of the large, flat gravis colonies. The minute colonies are either entirely black and opaque or have a dark center and light periphery. In diameter they may attain 1 mm, but are usually less and are often scarcely visible with the naked eye. The large colonies are about 2 to 3 mm in diameter. Interestingly enough the minute colonies may be either smooth and mitis-like in all respects but size, or rough and very much like gravis-type colonies except for their minuteness. It was especially interesting to observe that the majority of strains producing minute colonies are those described above as fermenting dextrose slowly, slightly or irregularly. The correlation between small colony production and defective dextrose fermentation is shown in Table II.

Such strains were found with increasing frequency from the beginning of this survey in 1942, onward. During this period of time "ordinary" cultures, *i.e.*, those not distinguished by minute colony formation, usually, though not invariably, reacted in the traditional manner in dextrose, *i.e.*, most of them fermented it promptly and regularly. For example, 21 ordinary cultures reacted as shown in Table III. Other large-colony strains studied at other times during this investigation gave similar results.

It appears that the type of diphtheria bacilli

characterized by minute colony formation and slow, slight or irregular dextrose fermentation is as distinct an entity as the gravis or mitis type and might, with equal or even greater propriety, be given the descriptive name *minus* for convenience in discussing it. The variability of *C. diphtheriae* under laboratory, and probably natural, conditions is well known and none of the forms hitherto described, including gravis and mitis types, have been found stable; nor is it likely that the *minus* type will prove an exception in this respect. Dextrose fermentation in these strains seems to be undergoing the actual process of changing from positive to negative or vice versa and very slight alterations in environment appear to affect the balance one way or the other. For example, strains tested for dextrose fermentation after subculture on blood agar acquired the property to ferment, especially in media containing casein hydrolysate. In routine pork-infusion broth, however, they remain defective for some time.

4. *Prevalence of minus type.* Although the *minus* type has not previously been noted as a distinctive variety it has nevertheless been observed in Baltimore a number of times during the past 8 years. Because of its rarity it had never been given special mention. In the interval between the last previous culture survey in Baltimore (Eastern Health District) in 1939 and the years covered by the present study it has become a relatively common[†] type. Furthermore, during these studies the *minus* variety of *C. diphtheriae* was prevalent in an epidemic in Baltimore which is elsewhere described.⁶ Since 1942 there has been a large influx into this city of population from other parts of the country, with whom many of the Baltimore children have mingled in school, and there has been a considerable increase in diphtheria morbidity and in the number of malignant cases in Baltimore. One wonders whether at least part of the increased diphtheria morbidity and fatality in Baltimore in the last year or so may not represent lack of experience of the human host with a hitherto strange variant of the parasite which, we know, readily alters itself in various respects.⁵

It is probable, as shown by Eller *et al.*,⁶ that over 70% of the school-age children in the area where the largest number of cases of diphtheria occurred had received toxoid immunization and notable that 64% of the cases occurred in children who had received toxoid. Inquiries have been made as to whether the *minus* type of organisms produces a toxin qualitatively different from that used in preparing the toxoid used in routine immunization. Complete studies have not yet been made but preliminary observations indicate that *minus* toxin is not appreciably different from other diphtheria toxins. The above-mentioned lack of adaptation and consequent unfavorable reaction on the part of the host would, then, appear to be related to the bacillary part of the diphtheria organism and not to its exotoxin and would suggest the desirability of considering the use of antibacterial or anti-endotoxic substances, in addition to anti-exotoxin alone, in both the prophylaxis and therapy of diphtheria. Experimental work in animals has already shown that such bacillary endotoxic antigens have distinct protective value.⁷

Summary. In Baltimore during the past 3 years the gravis type of diphtheria bacillus has, as heretofore, been absent or rare. However, during this period there have been isolated, chiefly from clinical cases of diphtheria and contacts thereof, but also from casual carriers, many virulent and nonvirulent diphtheria bacilli characterized by (a) the production of minute colonies on blood-agar media containing potassium tellurite, and (b) slow, slight or irregular fermentation of dextrose. These characters are very frequently found together and for such organisms the designation *minus* type is suggested as a convenience in discussion. Other virulent strains have been found which regularly ferment saccharose. The saccharose-fermenting strains studied do not appear to differ in other respects from ordinary diphtheria bacilli. A relationship between the *minus* type of *C. diphtheriae*, and recent epidemiological and clinical observations in Baltimore, as well as the possible value of bacterial antigens in addition to routine toxoid

⁶ Eller, C. H., and Frobisher, M., Jr., *Am. J. Hyg.*, 1945, in press.

⁷ Frobisher, M., Jr., and Parsons, E. I., *Am. J. Hyg.*, 1943, **37**, 53.

in prophylaxis, are discussed. Authors' summary.

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Johns Hopkins Hospital, and of Dr. J. Winfred Davis, Dr. C. Howe Eller, Dr. C. LeRoy Ewing and the members of their staffs, all of the Baltimore City Health Department, in collecting cultures and information for these studies. Valuable technical assistance was also given by Dr. Evelyn A. Mauss and Miss Elaine Updike of the Department of Bacteriology, Johns Hopkins School of Hygiene.

14944

Effect of Corn Grits on Nicotinic Acid Requirements of the Dog.*

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Pellagra has long been associated with the ingestion of relatively large amounts of corn. The early theories regarding this relationship included suggestions that corn contained toxic materials or acted as a carrier for pathogenic organisms. Goldberger and coworkers¹ clearly demonstrated that pellagra was caused by the deficiency of a dietary factor in certain foods commonly consumed by pellagrins, and in 1937² nicotinic acid was found to be the vitamin supplied in inadequate amounts. However, as analytical methods have been perfected, it has become apparent that on a dry weight basis, corn contains as much, or more, nicotinic acid as eggs, milk, polished rice, oats, and rye. Furthermore, Handler³ has found

that the blacktongue syndrome in dogs is more readily produced with diets containing corn than with purified rations which contain much less nicotinic acid. In addition, we have observed⁴ that corn exerts a marked growth retardation in rats which is counteracted by nicotinic acid therapy. Thus there is some characteristic of corn aside from its low nicotinic acid content, which tends to increase the organism's requirement for this substance.

Corn grits were used in the present study since they comprise a large portion of the dietary of many people in certain sections of this country, and since corn grits as prepared and marketed, usually contain significantly less nicotinic acid than does whole corn. The effect of corn grits on the nicotinic acid requirement of the dog was determined by feeding a nicotinic acid-low synthetic ration so modified as to include a high percentage of corn grits.

Experimental. The experiments were conducted with growing dogs which had been kept since weaning on the nicotinic acid-low synthetic ration reported in previous studies.⁵ All

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¹ Goldberger, J., and Tanner, W. F., *Pub. Health Rep.*, 1925, **40**, 54.

² Elvehjem, C. A., Madden, R. J., Strong, F. M., and Woolley, D. W., *J. Biol. Chem.*, 1938, **123**, 137.

³ Handler, P., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 263.

⁴ Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, 1945, **101**, 283.

⁵ Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **158**, 173.

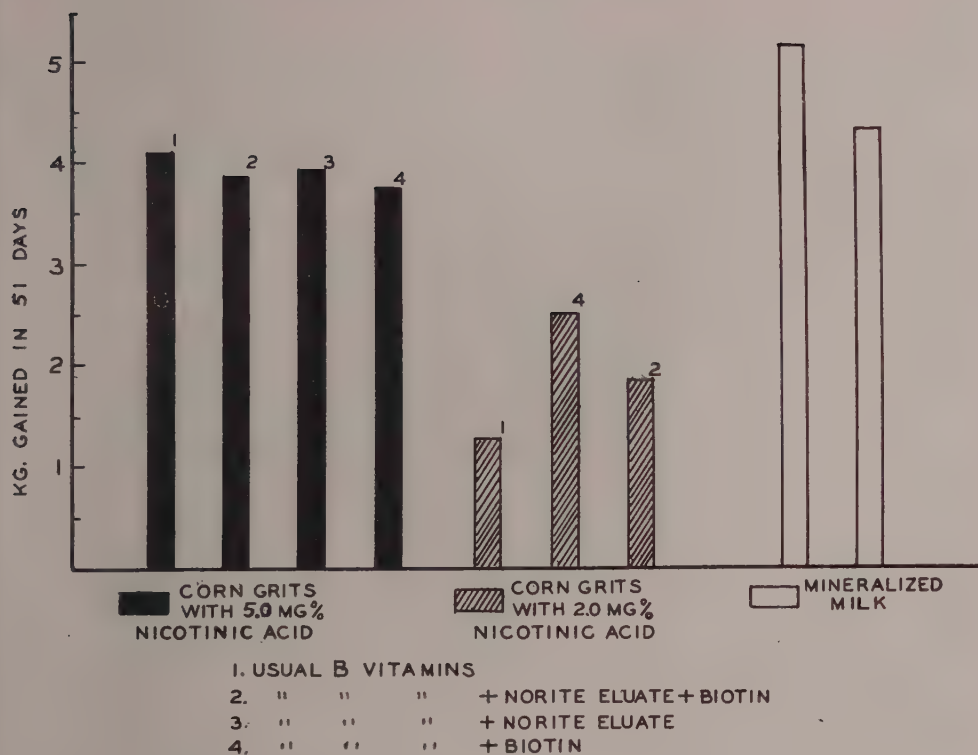


FIG. 1.

Growth of dogs on corn grits rations containing 2 levels of nicotinic acid.

of the dogs had undergone one or more attacks of blacktongue, but were in a good nutritional state with respect to nicotinic acid when placed on the experimental regime. Each dog was given 5 mg of nicotinic acid 1 week prior to starting the experiment.

The corn grits used and the synthetic basal ration were assayed microbiologically⁶ for nicotinic acid. The basal ration contained 0.064 mg %. Four lots of unenriched corn grits had 0.93, 0.56, 0.62 and 0.60 mg % of nicotinic acid while one sample each of whole white corn and whole yellow corn contained 2.25 and 2.28 mg % respectively, which is a fair average for whole corn of either variety. Two lots of enriched corn grits contained 4.9 and 6.1 mg % of nicotinic acid which is considerably higher than the average whole grain value. In all cases, unless otherwise indicated, the corn grits were incorporated into the synthetic

ration at a level of 60% at the expense of sucrose. This procedure insured an adequate protein level of about 24% for the entire ration. Generous amounts of the usual B vitamins except nicotinic acid (*i.e.*, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, and choline chloride) were fed by pipette. The ration was fed *ad libitum*. Vitamins A and D were given by dropper in the form of halibut liver oil.

The nicotinic acid content of the corn grits in the ration fed to one group of 4 dogs was adjusted by adding enough nicotinic acid to allow a level of 2.0 mg %, whereas the nicotinic acid content of the corn grits in the ration fed a second group of 3 dogs was increased in a like manner so as to contain 5.0 mg %. These two levels conformed to the nicotinic acid present in whole corn and enriched corn grits. Based on a daily food consumption of 35 g per kg, the two groups of

⁶ Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 471.

dogs received 0.42 and 1.05 mg of nicotinic acid per kg of body weight per day respectively. The lowest level of nicotinic acid fed therefore was at least 25% higher than the minimum requirement for the growing dog as established by Schaefer *et al.*⁷ using a synthetic ration. The two groups of dogs were kept on experiment for 51 days. The results (Fig. 1) show that the average growth for the 4 dogs on the higher level of nicotinic acid was 3.9 kg for the total period or 77 g per day, while the group average at the lower level was 1.88 kg or about 37 g per day. As a matter of comparison, 2 similar dogs on a mineralized whole milk diet grew an average of 4.75 kg in a like period of time. Whole milk contains approximately 0.7 mg % of nicotinic acid on the dry weight basis.

Since the norite eluate appears to be involved in the blacktongue syndrome in dogs⁵ and since biotin has been indicated as a vitamin for the dog,⁸ the effect of these vitamins, either alone or in combination was tested by supplementing these materials as indicated in Fig. 1. The growth results indicate no significant effect from either of these supplements and it appears that the nicotinic acid level is the predominating factor involved.

In an additional experiment, 2 dogs were placed at weaning on the corn grits ration in which the nicotinic acid level of the corn grits was adjusted to 1.0 mg % (the upper range for unenriched corn grits). This would furnish a daily calculated intake of about 0.21 mg of nicotinic acid per kg of body weight. Both of these dogs lost weight and died in 29 and 34 days.

In a final experiment the ration fed contained only 36% of corn grits (at the expense of sucrose) instead of the usual 60%. The corn grits in this case were enriched to such a level that the entire ration contained the equivalent of 1.6 mg % nicotinic acid as compared to 3.0 mg % for the previous 60% enriched corn grits ration. This ration was fed for a period of 30 days, during which time the dog gained 1.95 kg. This is a growth rate of about 65 g per day which is quite comparable

to the 77 g per day average obtained with dogs receiving corn grits containing the higher level of nicotinic acid. Thus when the level of corn grits was reduced from 60 to 36% one half the amount of nicotinic acid gave approximately the same growth as was previously observed.

Discussion. The evidence which is accumulating, such as Aykroyd's study with humans,⁹ Handler's findings with dogs,³ our observations with rats,⁴ and the present report on dogs indicates that corn significantly increases the nicotinic acid requirement. This deleterious effect might be explained in a number of ways.

On synthetic diets, there may be some beneficial synthesis of nicotinic acid which is diminished by the addition of corn grits; a substance (or substances) may be present in corn which combines with nicotinic acid to make it unavailable, or compounds may be present in corn which compete with nicotinic acid or its amide in body tissues. Koser¹⁰ has demonstrated that a number of bacteria destroy nicotinic acid and it is possible that corn may promote and support a flora which includes such microorganisms in large numbers. Regardless of the explanation for the deleterious effect of corn we have shown that the addition of adequate amounts of nicotinic acid completely counteracts this untoward action.

While it appears that corn tends to increase the nicotinic acid requirement, foods such as milk, which contain much less nicotinic acid, tend to decrease the requirement. This is undoubtedly due to the establishment of an intestinal flora which favors the synthesis of nicotinic acid.

Two approaches to the solution of the problem of corn as it affects the nicotinic acid requirement might be to enrich corn grits and other milled corn products with nicotinic acid considerably above the whole grain level, or, as Burkholder suggests,¹¹ by the development of varieties of corn which are markedly higher in their nicotinic acid content.

⁹ Aykroyd, W. R., and Swaminathan, M., *Ind. J. Med. Res.*, 1940, **27**, 667.

¹⁰ Koser, S. A., and Baird, G. R., *J. Inf. Dis.*, 1944, **75**, 250.

¹¹ Burkholder, P. R., McVeigh, I., and Moyer, D., *Yale J. Biol. Med.*, 1944, **16**, 659.

⁷ Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 679.

⁸ Smith, S. G., *Science*, 1944, **100**, 389.

Summary. When unenriched corn grits are incorporated in a synthetic ration to the extent of 60% of the sucrose, the nicotinic acid requirement of the dog is markedly increased and good growth does not result until the nicotinic acid content of the corn grits is in-

creased to about 5.0 mg % (more than 5 times the unenriched level) which furnishes the dog with about 3 times as much nicotinic acid as is required for comparable or better growth on a synthetic or whole milk ration.

14945

Electrical Activity of Acetylcholine at the Phase Boundary Between Cholesterol and Sodium Chloride Solution.

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In previous papers^{1,2} we have reported that acetylcholine chloride added to physiological salt solution produces a potential difference between that solution and various oils (nitrobenzene, cresol, guaiacol, etc.) so that the negative pole is on the side of the acetylcholine added.

In the apparatus an oil layer separates 2 layers of 0.9% NaCl solution. The experiments are attempts to duplicate in a simple model the potential differences found in the nervous system. Our object is to explain the potential that gives rise to the nerve impulse.

In the models previously described^{1,2} the oils used were chemically different from the lipids of the plasma membrane of the living

nerve. The electrical measurements with our oil cell technic cannot be done with pure cholesterol directly since this is a solid. The cholesterol must be dissolved in a water-immiscible yet conducting solvent which must not itself establish an electric negativity with acetylcholine. Such solvents are hard to find. It was found that benzyl alcohol gave no potential with acetylcholine in concentrations as high as 2%. In other words, no potential difference was measured between the two interfaces of benzyl alcohol and sodium chloride solution when acetylcholine was added to the latter on one side.

The low solubility of cholesterol in benzyl alcohol (precipitation occurs at concentrations above 0.6%) may explain the small magnitude of the acetylcholine potentials in these mixtures (Table I). It is a remarkable fact that 0.05% acetylcholine can produce a negativity of 6 millivolts at a phase boundary between a saline and an oil layer which is 99% inert (benzyl alcohol).

The nervous system contains many other lipids besides cholesterol—especially lecithin. It was found that 4% lecithin dissolved in the inactive solvent benzyl alcohol produced an electric negativity of 13 millivolts when 0.5% acetylcholine was added to the saline at one side of the oil layer. Unfortunately lecithin emulsifies in the aqueous layers, which makes the potential difference unstable.

The experiments described above demon-

TABLE I.
Phase Boundary Potential Difference Between Acetylcholine in Saline Solution in Contact with Benzyl Alcohol Containing 0.6% Cholesterol and Pure Saline.

Cone. of acetylcholine %	Potential difference between interfaces. Oil in contact with saline containing acetylcholine is negative.
0.05	6.0 millivolts
0.10	10.0 "
0.15	14.0 "

All experiments were performed at room temperature (25°C).

¹ Beutner, R., and Barnes, T. C., *Science*, 1941, **94**, 211.

² Beutner, R., and Barnes, T. C., *Biodynamica*, 1942, **4**, 47.

strate the electrical activity of acetylcholine at a phase boundary, which may explain the electrical phenomena in cholinergic nerves. These findings have theoretical interest and suggest that acetylcholine is more than a "chemical mediator" or "synaptic transmitter." Acetylcholine is capable of producing variations of electrical potential differences in the nervous system of a size and sign (invariably negative) similar to the electrical cur-

rents associated with the "nerve impulses."

Conclusion The addition of acetylcholine to saline solution in contact with cholesterol dissolved in an inert solvent (benzyl alcohol) gives rise to a negative electrical potential difference. It is suggested that this electrical negativity may play an important role in the origin of the negative variation associated with the nerve impulse.

14946

Locomotor Reactions of *Fundulus* Embryos with Abnormal Mauthner's Neurones.

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Some *Fundulus heteroclitus* embryos have been prepared in which the central nervous system is characterized by various deviations from normal in both the number and arrangement of Mauthner's neurones. In view of the divergent opinions expressed in the literature¹⁻³ concerning these cells, it has seemed desirable to investigate the behavior of these embryos for information concerning the function of the giant neurones.

The operations, which involved the implantation of grafts into the prospective brain region of gastrula stages, were performed by methods already described.⁴ The operated embryos were allowed to develop through to swimming stages, and their reactions were observed at least twice daily during the progress of development. The embryos were fixed in

Bouin's solution, sectioned at 10 micra, and impregnated with silver by a modification of Bodian's method.

In 7 embryos Mauthner's axones were lacking in the posterior medulla and cord. Four of these embryos, although capable of swimming both spontaneously and in response to artificial stimulation, were abnormally passive and tended to remain immobile, if left undisturbed, for long periods of time. The 3 remaining embryos exhibited vigorous movements. One of these was kyphotic because of disturbance to portions of the brain other than Mauthner's neurones, to such a degree that it was mechanically unable to maintain its normal postural relationships. Yet even in spite of the great mechanical obstacle to normal locomotion, it made continual efforts to produce the swimming reaction.

Four embryos showed one Mauthner's axone coursing posteriorly through the cord, instead of 2. In one of these the fiber extended down the cord only as far as the posterior trunk region, and was located in the dorso-lateral white rather than in its usual ventral position; this embryo, like some of those described above, was abnormally passive and at times immobile. In the 3 remaining embryos the axone ran in its usual position in the cord: one of these

* All operations were performed at the Osborn Zoological Laboratory, Yale University. Part of the histological work was completed in that laboratory during the tenure of a John Simon Guggenheim Memorial Foundation Fellowship.

¹ Bartelmez, G. W., *J. Comp. Neur.*, 1915, **25**, 87.

² Detwiler, S. R., *J. Exp. Zool.*, 1933, **64**, 415.

³ Coghill, G. E., *Psych. en Neur. Bladen*, 1934, **38**, 386.

⁴ Oppenheimer, J. M., *J. Comp. Neur.*, 1941, **74**, 131.

embryos was abnormally passive and immobile for long periods, although capable of swimming normally when artificially stimulated; the other 2 showed no evidence of atypical suppression of activity.

One embryo, of another category, whose swimming movements were normally sustained and vigorous, had an ectopic Mauthner's neurone whose axone coursed posteriorly only as far as the mid-trunk level; the contralateral axone was normal in the cord. This embryo veered to the left when swimming, but its deviation was accounted for not by changes in the central nervous system but rather by a pathological puckering of the skin in the trunk region which resulted in a contraction of the somites in the region involved.

There were 7 embryos in which the 2 Mauthner's fibers coursed posteriorly on the same side of the cord rather than on opposite sides. One of these was a kyphotic embryo which swam only sporadically; another was an abnormally immobile and passive embryo. The remaining 5 embryos exhibited no irregularities of locomotion attributable to the unusual location of the Mauthner's axones; 2 of them, in fact, were completely normal in behavior despite other structural abnormalities of the brain.

Finally, the cords of 22 embryos were characterized by the presence of axones arising from supernumerary Mauthner's cells. In some cases the extra axones were located dorso-laterally in the cord; in others they were located in their typical position. The arrangement of axones in these embryos was sometimes symmetrical, sometimes asymmetrical. The group included, for instance, among the embryos in which the fibers were in their normal position, 8 in which there was one axone on one side of the cord and 2 on the opposite side. In 2 of these embryos the only detectable abnormality of the brain was a unilateral duplication of Mauthner's cell with all its processes; in both cases the accessory cells and their dendrites were immediately adjacent to their normal counterparts and hence presumably subject to the reception of impulses from all the pathways usually impinging on the neurones. There were 3 embryos with one fiber on one side of the cord and 3 on the

opposite side; one in which there were 2 fibers on each side of the cord; one in which there were 3 fibers on one side of the cord and 2 on the other, all in the normal position.

One embryo in the group with supernumerary cells and fibers was abnormally passive and sometimes immobile. In all the other cases, the only variants of behavior recorded were such as could be attributed to abnormalities elsewhere in the brain than in Mauthner's system; in no case was the asymmetry in the arrangement of fibers reflected in asymmetry of reaction.

Discussion. The embryos described as inactive to an abnormal degree may perhaps correspond to the amphibian embryos in which one or both Mauthner's cells were extirpated, or in which the axones of the cells have taken an anterior rather than a posterior course, as described by Detwiler.^{5,6} The results reported here differ from Detwiler's, however, in that the present embryos lacking one or both axones in the cord were less uniform in behavior than Detwiler's, since only about half of them were abnormally passive or immobile.

This discrepancy may perhaps be explained by the fact that the operations on the *Fundulus* embryos were performed at earlier stages than were used in Detwiler's experiments. It is conceivable that a greater degree of regulability of function can be attained by a brain abnormal from its earliest formation than by one operated during stages after the neural tube is established. The apparently negative results of the *Fundulus* experiments in which supernumerary axones are present, and the absence of asymmetry in behavior in both fish and amphibian embryos asymmetrical as to Mauthner's axones in the cord, demonstrate the great regulability of function that can occur in developing brains. Other studies on *Fundulus* embryos, still in progress, show that brains rendered highly abnormal in structure by operations during gastrula stages can regulate functionally to a surprising extent.

The fact that the unilateral addition of one or more giant axones is not expressed in asymmetry of behavior brings up several other significant issues. Interesting physiological

⁵ Detwiler, S. R., *J. Exp. Zool.*, 1927, **48**, 15.

⁶ Detwiler, S. R., *J. Exp. Zool.*, 1943, **94**, 169.

problems, hardly germane to the present considerations, are raised by the demonstration of a condition whereby small slowly conducting fibers on one side of the central nervous system can balance the conduction of impulses by larger rapidly conducting contralateral fibers well enough to maintain symmetrical activity of the organism as a whole. The described condition is an important one, however, in relation to future analyses of the function of parts of the developing central nervous system. Any such analyses must take into account the degree to which, and the mechanisms by which, the embryonic brain can successfully compensate for the addition of supernumerary units.

Summary. Locomotor reactions have been observed in *Fundulus heteroclitus* embryos abnormal in number and arrangement of Mauthner's neurones. Five out of 11 embryos lack-

ing Mauthner's axones on one or both sides of the cord were abnormally passive or immobile; the remaining six exhibited vigorous swimming movements. Out of 7 embryos in which the 2 Mauthner's fibers coursed posteriorly both on the same side of the cord, 2 were abnormally passive and 5 exhibited vigorous movements. Axones from supernumerary Mauthner's cells were present in the cord in 22 embryos; one of these embryos was abnormally passive, but the others exhibited no variations of behavior attributable to the altered number of Mauthner's fibers. The apparently negative results of the experiments in which supernumerary axones were added, and the fact that asymmetry in the arrangement of fibers was not reflected in asymmetry of reaction, demonstrate a high degree of regulability of function in the developing brain of *Fundulus*.

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Activity of Pyridoxine Derivatives in Chick Nutrition.*

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The discovery¹ and confirmation² of the existence of pseudo-pyridoxine, which has very high bacterial potency, raises a question re-

garding its activity in animal nutrition. Carpenter *et al.*³ reported preparations of pseudo-pyridoxine to be less active for the rat than the original pyridoxine. We wish to report in this paper similar studies on the activity of pseudo-pyridoxine in chick nutrition.

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[‡] Called pyracin-5 in this paper to distinguish it from the lactone of 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine, which has also been reported to be active.⁷ Pyracin-5 has also been termed α -pyracin and pyridoxie acid.

¹ Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

² Carpenter, L. E., and Strong, F. M., *Arch. Biochem.*, 1944, **3**, 375.

³ Carpenter, L. E., Elvehjem, C. A., and Strong, F. M., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 123.

TABLE I.
Composition of Rations.

Constituent	494	494-B ₆	494K
Dextrin* (g)	61	61	61
Cascint (g)	18	18	18
Gelatin (g)	10	10	10
Salts V (12) (g)	6	6	6
Soybean oil (g)	5	5	5
1(-) Cystine (g)	0.3	0.3	0.3
Thiamine HCl (mg)	0.3	0.3	0.3
Riboflavin (mg)	0.6	0.6	0.6
Calcium pantothe- nate (mg)	2	2	2
Choline Cl (mg)	150	150	150
Nicotinic acid (mg)	5	5	5
Pyridoxine HCl (mg)	0.4		0.4
Biotin (mg)	0.02	0.02	0.02
i-Inositol (mg)	100	100	100
B ₁₀ and B ₁₁ concen- trate (mg)†	100	100	
Vitamin D ₃ (mg)	0.004	0.004	0.004
α-Tocopherol (mg)	0.03	0.03	0.03
2-Methyl-1, 4-naph- thoquinone (mg)	0.05	0.05	0.05
Vitamin A (U.S.P. units)	1700	1700	1700

* Made by adding corn starch to water until the consistency of putty is obtained, autoclaving for 3 hr drying and grinding.

†SMACO vitamin test casein.

‡ Made as described for the superfiltrol eluate.¹²

When the two microbiologically potent derivatives of pyridoxine, pyridoxal (2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine) and pyridoxamine (2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine), became available in crystalline form^{4,5} it seemed desirable to test them for vitamin B₁₀ and B₁₁ activity as well as for vitamin B₆ activity in the chick. Snell⁶ has recently reported these compounds to be slightly more active than pyridoxine in rat nutrition. Pyracin-5,[‡] the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine, was included in these tests because Scott *et al.*⁸ found it to be active in chick nutrition. Bound forms of pyridoxine which have been found to be active

for both the rat and bacteria⁹ are not considered here.

Symptoms of the vitamin B₆ deficiency syndrome in the chick, not previously described, are also reported.

Experimental. The methods used were similar to those previously described.¹⁰⁻¹² Day-old White Leghorn chicks, obtained from the University Poultry Department or commercial hatcheries, were placed in heated cages with raised screen bottoms and fed basal ration for three days. They were then divided uniformly into groups of 6 and fed (*ad libitum*) the basal ration with the proper supplement added.

The composition of the different rations used is given in Table I. The vitamin B₆ activity of the different preparations was tested by using ration 494-B₆. Likewise, the ability of the compound to replace vitamins B₁₀ and B₁₁ was determined on ration 494-K. The compounds were also added to a ration containing the vitamin B₁₀ and B₁₁ concentrate and the response noted.

Blood samples for prothrombin estimation were drawn by heart puncture from a few of the chicks at 2, 3 and 4 weeks. Recovery from this procedure appeared rapid since the blood picture of these chicks on the following week was very similar to that of other chicks within the same group. Whole blood clotting time was determined by the capillary method.¹³

Results. The data in Table II show that the addition of pyridoxal, pyridoxamine, or pyracin-5 to the vitamin B₁₀ and B₁₁ deficient diet has no effect on growth or feathering in the chick. Pyridoxamine is also inactive when fed in a mixture with xanthopterin,[§] thymine and guanine. Groups 9-12 inclusive indicate that

⁹ Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, 1944, **155**, 119.

¹⁰ Briggs, G. M., Jr., Mills, R. C., Hegsted, D. M., Elvehjem, C. A., and Hart, E. B., *Poultry Sci.*, 1942, **21**, 379.

¹¹ Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **158**, 303.

¹² Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1944, **153**, 423.

¹³ McGowan, J. P., *Brit. Med. J.*, 1907, **2**, 1580.

§ Prepared by Dr. R. C. Mills, now in the service of the armed forces.

⁴ Snell, E. E., *J. Biol. Chem.*, 1944, **154**, 313.

⁵ Harris, S. A., Heyl, D., and Folkers, K., *J. Biol. Chem.*, 1944, **154**, 315.

⁶ Snell, E. E., *Abstracts Am. Chem. Soc. Meeting*, New York, 1944, p. 7B.

⁷ Scott, M. L., Norris, L. C., Heuser, G. F., and Bruce, W. F., *J. Am. Chem. Soc.*, 1945, **67**, 157.

⁸ Scott, M. L., Norris, L. C., Heuser, G. F., Bruce, W. F., Coover, H. W., Jr., Bellamy, W. D., and Gunsalus, I. C., *J. Biol. Chem.*, 1944, **154**, 713.

TABLE II.
 Results on the Vitamin B₁₀ and B₁₁ Deficient Ration.

No.	Supplements/100 g of Ration 494K	Dead/chicks	B ₁₀ * %	B ₁₁ † %	4 wks avg wt g
1	None	1/18	30	0	125
2	500 γ pyridoxine	2/12	30	14	139
3	50 γ pyridoxal	0/6	20	—17	108
4	100 γ "	1/5	45	—35	90
5	50 γ pyridoxamine	0/6	30	—7	118
6	500 γ "	2/12	30	14	139
7	50 γ " + 50 γ xanthopterin + 100 γ thymine + 100 γ guanine	1/6	25	3	128
8	500 γ pyracin-5	0/11	35	5	130
9	100 mg vit B ₁₀ , vit B ₁₁ concentrate (control diet)	0/18	100	100	225
10	100 mg vit B ₁₀ , vit B ₁₁ concentrate + 200 γ pyridoxine	0/6	100	103	228
11	100 mg vit B ₁₀ , vit B ₁₁ concentrate + 200 γ pyridoxamine	0/6	100	112	237
12	100 mg vit B ₁₀ , vit B ₁₁ concentrate + 200 γ pyracin-5	0/5	100	85	210

* Feathering scale: 0 = very poor; 25 = poor; 50 = fair; 75 = good; 100 = very good.

† Growth results expressed as % or g gain over basal $\times 100$
control weight—basal weight

pyridoxine and pyracin-5 give no significant response when fed with the complete ration. These compounds produced no consistent effect on hemoglobin formation.

The growth and survival data from 3 vitamin B₆ experiments, Table III, indicate that pyridoxal is slightly more than one-half and pyridoxamine is about four-fifths as active as pyridoxine, while pyracin-5 is completely inactive. In one experiment pyridoxal and pyridoxamine were fed at a very low level to eliminate the possibility that they might be toxic at the higher levels fed previously. The results of groups 7 and 9 indicate that these compounds have no pseudopyridoxine-like activity in the chick.

B₆ Deficiency Syndrome. Most of the symptoms previously reported,¹⁴⁻¹⁶ for vitamin B₆ deficient chicks were observed. These include reduced food consumption and growth, asthenia, poor feather development (resembling that obtained in an arginine-glycine deficiency), occasional fits and convulsions, and death. Hyperexcitability, reported by

Lepkovsky¹⁶ as stage I, was not observed although strong stimuli were applied, *e. g.*, various loud noises, sudden movements, and short air blasts.

The onset of a fit (whether mild or ending in convulsions) is usually accompanied with short, high-pitched chirps. Hemoglobin values (Table IV) indicate that vitamin B₆ deficiency in chicks is accompanied by a definite anemia. The anemia is quite pronounced in some of the deficient chicks while in other chicks a dehydration seemed to have occurred which manifested itself as a hemoglobinemia.

While making hemoglobin determinations, difficulty in preventing clotting was unexpectedly encountered in some of the vitamin B₆ deficient chicks. Upon further investigation it was found that the deficient chicks (only 3 were alive at this time) had a whole blood clotting time of 2.5 (1.8-4.0) min, while the value for one of the controls was 4.5 min and that of 2 others was greater than 10 min. The results of the next experiment indicated that 2-week-old chicks have similar whole blood clotting times, while 3- and 4-week-old chicks, deficient in vitamin B₆, have a decreased clotting time (when compared with the control chicks). At 4 weeks it was found (Table V) that 25 γ of pyridoxine supplement per 100 g of ration 494-B₆ gave no response,

¹⁴ Hegsted, D. M., Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1939, **130**, 423.

¹⁵ Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 180.

¹⁶ Lepkovsky, S., and Kratzer, F. H., *J. Nutrition*, 1942, **24**, 515.

TABLE III.
 Growth Results Using Vitamin B₆ Deficient Ration.

No.	Supplement per 100 g 494-B ₆	Weight g at 4 wks	Approximate activity†	Dead/chicks
1	None	69		14/24
2	5γ pyridoxine HCl	3* wks	1	6/6
3	25γ "	74	1	0/6
4	50γ "	108	1	1/11
5	100γ "	197	1	0/12
6	400γ "	222	1	0/24
7	5γ pyridoxal HCl	2* wks	low	6/6
8	50γ "	95	.6	3/6
9	5γ pyridoxamine HCl	2* wks	low	6/6
10	50γ "	127	.9	3/6
11	100γ "	167	.8	0/6
12	100γ pyracin-5	50	0	5/6
13	200γ "	3* wks	0	6/6
14	400γ "	4* wks	0	6/6
15	As 14 + 100γ pyridoxine HCl	138	no effect	0/6

* Indicates time of death of the last chick in the group.

† Activity values are based on survival data as well as growth data in groups No. 8 and No. 10.

50γ gave an intermediate value and chicks receiving 100γ had clotting times comparable to the controls. Chicks which received 100γ of pyridoxine per 100 g of ration for the first week and the vitamin B₆ deficient ration for the next 3 weeks (group 5) had a short whole blood clotting time (2 min) at 4 weeks. Control chicks receiving 5% alfalfa or 1 mg of 2-methyl-1, 4-naphthoquinone per 100 g of ration 494 (groups 7 and 8 respectively) had comparatively long whole blood clotting times.

Determinations of the prothrombin level (or activity) (Table V) made by Dr. John B. Field indicate that the decrease in whole blood clotting time is accompanied by a detectable hyperprothrombinemia in the B₆ deficient chicks.

No gross pathology of the internal organs was observed at autopsy although the spleens of the vitamin B₆ deficient chicks appeared abnormally small. Further investigation indicated that the spleens of the vitamin B₆ chicks were proportionately smaller than those of control chicks (the spleens were respectively 0.09 and 0.20% of the body weights).

Discussion. Since the activities found for pyridoxal and pyridoxamine using the chick assay are not in accord with the high bacterial potencies, pyridoxine remains the most important of the B₆ vitamins in chick nutrition. Assays of natural materials for nutritionally active vitamin B₆ should thus be made using either biological methods, or corrections for the

 TABLE IV.
 Hemoglobin Values of 4-Week-Old Chicks.

Diet	No. chicks	Hb g/100 cc	Range
B ₆ deficient	10	6.5	(3.1- 9.7)
Complete	12	8.7	(6.4-10.4)
Difference		2.2	
Standard error = 0.68*			

* The statistical validity of these data was confirmed by Dr. S. Ames by use of "Students" *t*-test for unequal samples.

differences found between the biological and microbiological activities of compounds such as pyridoxine, pyridoxal and pyridoxamine.

Pyracin-5 appears inactive under all of the conditions used, in contradiction to the first report of the Cornell workers.⁸ A later report¹⁸ indicates that a concentrate in their ration was contaminated with some *L. casei* factor. Recently Scott *et al.*⁷ have reported that the lactone of both the 4- and the 5-carboxypyridine derivatives are active for the chick in the presence of a *Lactobacillus casei* factor. This finding, presented without data, is difficult to evaluate, but it should be pointed out that the third *Lactobacillus casei* factor gives a significant response without feeding any lactone.^{11,18} This response, as well as the

17 Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **38**, 1.

18 Norris, L. C., *Flour and Feed*, 1944, **45**, 44.

19 Huthings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, 1944, **99**, 371.

TABLE V.
 Whole Blood and Prothrombin Clotting Times of 4-Weeks-Old Chicks.

No.	Supplement/100 g 494-B ₆	No. chicks	Whole blood clotting time (min)	Prothrombin time (sec)*
1	None	3	4 (3.3- 5.0)	31 (28.0-32.2)
2	25γ pyridoxine	4	4 (3.0- 6.0)	
3	50γ "	4	8 (1.2-13.5)	
4	100γ "	4	17 (8.8-29.3)	
5	100γ " (1st week only)	2	2 (2.0- 2.5)	
6	400γ "	4	16 (14.5-19.0)	39 (33.9-46.3)
7	400γ " + 5 g alfalfa	4	21 (9.3-34.0)	
8	400γ " + 1 mg 2-methyl-1, 4-naphthoquinone	4	11 (6.3-13.8)	
9	100% Practical broiler ration	4	20 (10.5-29.0)	

* Determinations by Dr. John B. Field using the 12.5% plasma method¹⁷ with thromboplastin prepared from chick brain.

response to other compounds such as vitamin C²⁰ and p-aminobenzoic acid,²¹ is not obtained when the bacterial flora is altered by feeding sulfa drugs or corn meal.¹¹ While we have been unable to observe any effect due to the feeding of pyracin-5 it is entirely possible that certain responses may be obtained when it is

²⁰ Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1944, **55**, 130.

²¹ Briggs, G. M., Jr., Luckey, T. D., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1943, **52**, 7.

²² Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 194.

²³ National Research Council, *Recommended Nutrient Allowances for Domestic Animals*, 1944, Bull. No. 1, p. 3.

fed in combination with other factors.

The decreased whole blood clotting time, as well as the decreased prothrombin clotting time, observed in the vitamin B₆ deficient chicks is obtained with a ration containing adequate amounts of vitamin K.^{22,23} Whether the anemia and small spleens are correlated in some way with the blood clotting mechanism is not known.

Summary: Pyridoxal, pyridoxamine and pyracin-5 were found to be inactive singly in replacing vitamin B₁₀ or vitamin B₁₁ in chick nutrition. The biological vitamin B₆ activity of these three compounds is approximately $>\frac{1}{2}$, $\frac{4}{5}$, and 0 respectively. Vitamin B₆ deficiency symptoms in the chick were found to include a decreased clotting time, hyperprothrombinemia, small spleens and anemia.

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Effect of Growth Influencing Factors on the *In Vitro* Deamination of dl-Alanine.

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There is evidence concerning specific effects upon intermediary protein metabolism of hormonal factors capable of influencing growth (for review, see Long¹). Thus the growth fac-

tor of the pituitary has been shown to cause a fall in blood NPN, chiefly amino and urea N;² a decrease in total NPN in the body and especially in the liver, all nitrogenous fractions

* Aided by a grant from the Charlton Research Fund.

¹ Long, C. N. H., *Cold Spring Harbor Symposia on Quant. Biol.*, 1942, **10**, 91.

² Teel, H. M., and Walkins, O., *Am. J. Physiol.*, 1929, **89**, 662.

³ Shaffer, N. K., and Lee, M. O., *J. Biol. Chem.*, 1935, **108**, 355.

TABLE I.
Deamination of dl-Alanine by Rat Tissue Slices.

	Amino N* disappearance $\gamma/\text{mg dry wt/hr}$	Ammonia N* production $\gamma/\text{mg dry wt/hr}$	Urea N* production $\gamma/\text{mg dry wt/hr}$	QO ₂ *
Liver-Adult				
Normal—No injections	-1.6 ± 0.20 (15)	0.19 ± 0.03 (25)	0.30 ± 0.05 (25)	0.07 ± 0.11 (11)
Pituitary growth complex	-1.1 ± 0.29 (5)	0.17 ± 0.04 (15)	0.30 ± 0.04 (14)	1.1 ± 0.25 (5)
Adrenal cortical extract		0.03 ± 0.08 (15)	0.43 ± 0.11 (15)	
Thyroxin	-1.6 ± 0.43 (13)	0.13 ± 0.04 (15)	0.44 ± 0.02 (14)	1.2 (3)
" + growth compl.	-1.3 ± 0.11 (12)	0.13 ± 0.02 (16)	0.49 ± 0.04 (16)	
Thyrotropic hormone	-1.0 ± 0.04 (4)	0.11 ± 0.07 (23)	0.43 ± 0.14 (16)	
" +				
growth complex	-0.3 ± 0.21 (5)	0.17 ± 0.05 (16)	0.44 ± 0.16 (16)	
Kidney-Adult				
Normal—No inj.	-4.6 ± 0.27 (22)	2.65 ± 0.15 (31)	-0.12 ± 0.12 (23)	7.9 ± 0.64 (22)
Pituitary growth compl	-4.9 ± 0.31 (20)	2.59 ± 0.09 (38)	0.00 ± 0.06 (37)	8.6 ± 0.40 (22)
Adrenal cortical extr	-4.7 ± 0.73 (3)	2.04 ± 0.36 (14)	0.10 ± 0.31 (14)	8.5 (3)
Thyroxin	-4.8 ± 0.37 (6)	2.42 ± 0.13 (9)	-0.19 ± 0.04 (9)	
" + growth compl		2.15 ± 0.13 (7)	-0.06 ± 0.06 (7)	
Thyrotropic hormone		2.39 ± 0.21 (7)	0.04 ± 0.02 (7)	
" +				
growth compl		2.31 ± 0.15 (8)	0.05 ± 0.06 (8)	
Kidney-Young Animal				
Normal	-4.1 ± 0.27 (16)	2.32 ± 0.12 (27)	0.21 ± 0.07 (20)	6.8 ± 0.41 (14)
" + growth compl	-4.6 ± 0.33 (16)	2.38 ± 0.15 (27)	0.01 ± 0.16 (27)	7.0 ± 0.43 (9)
Hypophysectomized	-3.7 ± 0.70 (5)	1.44 ± 0.29 (5)	0.38 ± 0.18 (4)	6.5 ± 0.41 (5)
" +				
growth compl	-4.4 ± 0.94 (4)	1.66 ± 0.58 (4)	0.02 ± 0.07 (4)	6.5 ± 0.92 (4)
Adrenalectomized	-5.2 ± 0.25 (16)	2.17 ± 0.11 (16)	0.05 ± 0.05 (15)	6.2 ± 0.16 (12)
" +				
growth complex	-4.9 ± 0.24 (18)	1.92 ± 0.15 (19)	-0.21 ± 0.18 (18)	5.5 ± 0.43 (17)

* Difference between tissues in medium without substrate and with 0.01 M alanine. Figures in parentheses indicate the number of experiments.

\pm = Standard mean deviation.

participating except NH_3 ,³ and a decreased urinary N, chiefly urea.⁴ These findings have received later confirmation, but the nature and locus of the effects is still not clear. In the case of other hormones the results are even more obscure. The thyroid, at least at certain age levels, is concerned with growth, but evidence associating this with protein metabolism is scanty. Sternheimer⁵ observed increase in protein liver N following a single thyroxin injection, and Zitovskaya⁶ noted an increased synthesis by the liver of alanine from pyruvic acid and NH_3 , following thyroxin injection. As for the functions of the adrenal cortex in this connection, Russell and Wilhelmi⁷ described a lower than normal rate of deamina-

tion by the kidney of dl-alanine and 1 (+) glutamic acid, in adrenalectomized rats, with restoration of function on administering adrenal cortical extract; while Jimenez-Diaz⁸ reported a rather remarkable decrease in kidney QNH₃ as a result of adrenalectomy in the rat. On the other hand, in the functionally nephrectomized animal Evans⁹ found that the blood amino and urea N were unaffected by adrenalectomy.

In the following we report the fact that we have been unable, under the conditions of our experiments, to obtain any unequivocal evidence of an effect on the *in vitro* deamination of dl-alanine by various growth influencing factors.

Kidney and liver slices of adult and young (30-40-day-old) rats were used, the medium

⁴ Gaebler, O. H., *J. Expt. Med.*, 1933, **57**, 349.

⁵ Sternheimer, R., *Endocrinology*, 1939, **25**, 899.

⁶ Zitovskaya, F., *Bull. biol. med. exptl. U.S.S.R.*, 1939, **7**, 114.

⁷ Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, 1941, **137**, 713.

⁸ Jimenez-Diaz, C., *Lancet*, 1936, **2**, 1135.

⁹ Evans, G., *Endocrinology*, 1941, **29**, 737.

¹⁰ Canzanelli, A., Rogers, G., and Rapport, D., *Am. J. Physiol.*, 1942, **135**, 309.

being a previously described modified phosphate-buffered Ringer's solution.¹⁰ The hormones were injected as follows: (1) Pituitary Growth Hormone: 0.05 cc Ayerst, McKenna and Harrison Growth Complex[†] (5 Collip units) per 100 g wt intraperitoneally, once 2 days before the expt., twice (A.M. and P.M.) one day before, and once about 1 hr before (on the basis of the period of maximum N retention—M. Lee, personal communication); (2) Thyroxin: 1 mg (1 cc ampoule La Roche) subcutaneously 2 days before experiment (on the basis of the maximum effect on the metabolic rate of the intact animal); (3) Thyrotropic Hormone: Either our own preparation, made according to the method of Rowland and Parkes, 10 mg intraperitoneally on 5 successive days before experiment, or Armour Thyrotropic Factor, 0.2 cc intraperitoneally daily for 5 days (on the basis of the maximal metabolic effect); (4) Adrenal Cortical Hormone: 1 cc Upjohn Extract (2.5 rat units) every hr for 5 hr before the experiment; (5) Combinations of hormones were given according to the above dosages.

In the medium from which the tissue had been removed, amino N was determined by the method of Van Slyke and Neill and ammonia and urea by the method of Conway and Byrne.¹¹ The tissue was incubated in the medium with and without alanine at 37°C for 110 minutes. In most cases each recorded experiment represents the determination of the metabolism in the pooled media of 5 Warburg flasks, each of which contained 3 cc of medium and 30 to 40 mg dry-weight of tissue. In the remaining experiments, about 150-200 mg dry weight of tissue were incubated with 15 cc of medium in a single Erlenmeyer flask.

It will be seen in Table I that the NH_3 and

urea formation by adult liver in the presence of alanine were entirely unaffected by any previous injection. The amino N disappearance was always greater than could be accounted for by the NH_3 and urea, except in the experiments where thyrotropic and growth hormones were given together, but even here there was no apparent change in deamination.

The kidney, as Krebs¹² first showed, deaminated alanine much more rapidly than did liver; there was no evidence of urea formation; and the NH_3 did not account for the disappearance of amino N. In both adult and young kidney, as in liver, there was no unequivocal evidence in any of the experiments that either the injection of hormonal preparations, or adrenalectomy, had the slightest effect on the catabolism of the amino acid. In the case of the adrenalectomized animals our results are in conflict with those of Russell and Wilhelmi, quoted above. Even in the case of the hypophysectomized animal where a possible statistical case might be made for a lessened ability to deaminate alanine, we do not believe the evidence warrants such a conclusion.

Apart from the fact that decreased deamination may have occurred, but have been of an order of magnitude too small for measurement, the negative results may be due to (1) the fact that the catabolism of "non-essential" amino acids like alanine are unaffected by growth factors; (2) that protein synthesis may be independent of deamination, following rather than forestalling it, in which case one would have to assume that the synthesis is occurring elsewhere than in the tissue studied, else it would be observable by our technic. The foregoing factors require further study.

Summary. Experiments on the effect of hormonal factors capable of influencing growth have failed to yield evidence of a decreased deamination of dl-alanine by rat liver and kidney.

[†] We are indebted to Ayerst, McKenna and Harrison for a generous supply of their Growth Complex.

¹¹ Conway, E. J., and Byrne, A., *Biochem. J.*, 1933, **27**, 419, 430.

¹² Krebs, H. A., *Zeitsch. f. Physiol. Chemie*, 1933, **217**, 191.

14949

Significance of the Age Factor and Sex Glands in Experimental Leukemia of Mice.*

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In the course of experiments concerning the effect of the age factor on the development of estrogen cancers in the mammary glands of mice of strain D,¹ leukemia occurred in a large number of animals. The following report deals with our observations on the influence of age and sex glands on the development of leukemia in these mice.

Material and Methods. 258 mice (141 males and 117 females) of the closely inbred strain D were examined. The incidence of spontaneous leukemia in this strain was 2%. Seventy-three males and 57 females were castrated at the age of 2 to 3 weeks, 68 males and 60 females remained as non-castrated controls. All mice were injected subcutaneously with 200 Rat Units of estradiol benzoate in sesame oil[†] once a week for 5 months. The animals were divided into 2 groups: In Group A, estrogen was injected about a week after operation, *i.e.* at the age of 3 or 4 weeks; in Group B, the administration of estrogen was begun only 6 weeks or 3 months after castration. After discontinuation of the estrogen treatment, the animals were allowed to live until their natural death. Liver, spleen, lymphnodes and, if necessary, additional tissues were studied histologically, and in many cases suspect of leukemia blood smears were made at autopsy.

In the following table, columns 3 to 6 show results in age group A, columns 6 to 9 those in age group B, column 11 the percentage

of leukemia irrespective of the age at which the treatment was started. Columns 3 and 7 give the number of animals in each group, columns 4 and 8 the absolute number of cases, columns 5 and 9 the percentage of cases of leukemia, and columns 6 and 10, the mean age of the animals at the time of death.

Observations. In Group A, the incidence of leukemia in intact males receiving estrogen was 30%; in castrated males treated with estrogen it rose to 50%. In the corresponding females, the difference was less conspicuous and probably not of significance. The trend seemed reversed, intact females developing leukemia in 45.8% as compared with a 40% incidence in ovariectomized estrogen-treated animals.

A comparison of age groups A and B gives the following results: Neither in castrated males nor in ovariectomized females was the incidence of leukemia affected by the age factor; the same number of castrated animals developed leukemia, whether they were injected immediately after castration or at a later date. Likewise in females with intact ovaries, the difference in the two age groups was less marked and perhaps not significant: Leukemia was somewhat more frequent in females injected at 3 or 4 weeks of age (45.8%) than in females injected at the age of 2 to 3 months (36.1%). However, the age at the beginning of the treatment decidedly influenced the incidence of leukemia in non-castrated males. In this group, leukemia developed almost twice as often (57.9%), if estrogen was administered after sexual maturity than if it was given at an earlier age (30%).

Seven animals of group B developed lymphosarcomas, whereas none occurred in group A. Five of these tumors were seen in intact males and only one each in an ovariectomized and

* The experiments were carried out in the Department of Pathology, New York University, College of Medicine, New York City. Aided by the David May-Florence G. May Fund.

¹ Loeb, L., Suntzeff, V., Burns, E. L., and Schenken, I. R., *Arch. Pathol.*, 1944, **38**, 52.

[†] We are indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J., for the generous supply of Progynon B.

TABLE I.
Incidence of Leukemia in the Two Age Groups.

	Group A					Group B				Total group A and B %
	No. of animals	No. of animals	No. of cases of leuke- mia	% leuke- mia	Age at death mo	No. of animals	No. of cases of leuke- mia	% leuke- mia	Age at death mo	
♂ controls	68	30	9	30.0	11.6	38	22	57.9	12.6	45.6
♂ castrates	73	30	15	50.0	11.3	43	22	51.2	11.7	50.7
♀ controls	60	24	11	45.8	11.3	36	13	36.1	13.3	40.0
♀ ovariectomized	57	20	8	40.0	12.0	37	14	37.8	11.7	38.6

in an intact female.

Our observations in the two age groups suggest that (1) Orchiectomy increases, (2) ovariectomy perhaps slightly decreases the incidence of leukemia, if estrogen treatment is begun before the age at which sexual maturity occurs, (3) these differences in the incidence of leukemia do not exist, if estrogen is administered at or after the time of sexual maturity. The presence of the testicle in sexually immature males seems somehow to inhibit the development of leukemia which would occur under the influence of estrogen.

In spite of the limited number of animals in the various groups, our results agree with those of other investigators and seem to clear up some discrepancies between their conclusions. Whereas McEndy, Boon and Furth² found ovariectomy to decrease and orchidectomy slightly to increase the incidence of leukemia in the high leukemia stock Ak, Murphy³ reported that ovariectomy hardly affected but that orchidectomy markedly increased the incidence of leukemia in the high leukemia strain RIL. In discussing his findings,

Murphy suggested that the age of the animal at which castration was performed might account for the differences between his results and those of McEndy, Boon and Furth.

In our experiments, a low leukemia strain was used; the animals were castrated at the same age, but estrogen injections were begun at different ages. The results obtained in the two different age groups seem to support Murphy's suggestion. If the age factor is not considered and the incidence of leukemia computed with disregard of the age groups (Col. 11), the effect of castration is obscured, the differences between castrates and non-castrates being as low as 5.1% in males and 1.4% in females.

Conclusions. Male mice of strain D treated with estrogen after sexual maturity developed leukemia more readily than those injected from an earlier age on. In castrated males, the incidence of estrogen induced Leukemia was significantly increased over that in non-castrated animals, if the treatment was started at an early age. In females receiving estrogen from an early age on, ovariectomy slightly decreased the incidence of leukemia, and in those treated after sexual maturity, no effect of ovariectomy was noted.

² McEndy, D. P., Boon, M. C., and Furth, J., *Cancer Research*, 1944, **4**, 377.

³ Murphy, J. B., *Cancer Research*, 1944, **4**, 622.

Effect of Ovariectomy on Basal Metabolic Rate of the Albino Rat.

CLARENCE R. MOTT. (Introduced by Con Fenning.)

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Changes in the over-all body weight, and also the changes in the weights of various organs, following ovariectomy in the young albino rat, have been carefully studied, reported, and the literature reviewed by Freudenberg *et al.*¹⁻⁴

With respect to the metabolic changes, Hemmingsen,^{5,6} after reviewing the literature, states that the influence of ovariectomy on the metabolism of the rat has not been solved. Hemmingsen has reported the results of his own work on the effects of ovariectomy on metabolism of the rat and concluded that if there is a difference in the metabolic rate of the ovariectomized and control animal it is indeed slight.

In view of the results on growth reported by Freudenberg *et al.*, cited above, it would seem that one should reasonably expect a significant difference in the basal metabolic rate of the ovariectomized rats as compared with the controls if the comparisons were made at the proper time following ovariectomy. The physiological phenomenon associated with the marked acceleration in growth rate up to about 100 days in the ovariectomized animal should be accompanied by an increase in metabolism.

Experimental Procedure. Rats were selected at random, weaned and ovariectomized under ether anesthesia at the 26th day of age. Litter mates were used as controls. A laparotomy

was performed on the control animals, the ovaries being examined but not removed. Immediately following the operation the animals were placed in a constant temperature room in which the temperature was maintained between 28 and 29°C.

All metabolic measurements were made between 28-29°C as this temperature,⁷⁻⁹ is generally considered to be the critical temperature for the rat. A metabolimeter very similar to that used by Tainter,¹⁰ was employed.

In the biometrical analysis of the results to determine significance of changes the method given by Fisher,¹¹ for testing for the significance of two means was used. Rubner's¹² formula was used in calculating the surface area of the animals.

Observations and Discussion. A statistical comparison of body weights (Table I) clearly shows that although the test animals were on the average heavier in all 4 age groups, the difference was not significant in the 40-60 day group, definitely significant in the 90-105 day group, only probably significant in the 175-210 day group, and not significant in the 800-840 day group.

The results in the first 3 age groups, with respect to changes in body weight following ovariectomy, compare favorably with those reported by Freudenberg *et al.*, as cited above. These authors did not extend their weight curves to include the older animals.

In the present study the average weight of the ovariectomized rats was at a maximum about the 40th week and then gradually de-

¹ Freudenberg, C. B., and Billeter, Oscar A., *Endocrinology*, 1935, **19**, 345.

² Freudenberg, C. B., and Howard, Philip M., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **36**, 144.

³ Freudenberg, C. B., and Hashimoto, E. I., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 530.

⁴ Freudenberg, C. B., and Hashimoto, E. I., *Am. J. Anat.*, 1937, **62**.

⁵ Hemmingsen, A. M., *Skand. Arch. Physiol.*, 1933, **67**, 137.

⁶ Hemmingsen, A. M., *Skand. Arch. Physiol.*, 1934, **68**, 89.

⁷ Goto, Kiko, *Biochem. Z.*, 1923, **135**, 107.

⁸ Wesson, L. G., *J. Nut.*, 1931, **3**, 503.

⁹ Sherwood, T. C., *J. Nut.*, 1936, **12**, 223.

¹⁰ Tainter, M. L., and Rytand, David A., *PROC. SOC. EXP. BIOL. AND MED.*, 1934, **32**, 361.

¹¹ Fisher: *Statistical Methods for Research Workers*, 5th Ed., 1934.

¹² Rubner, M., *Leipzig*, 1902, 281.

TABLE I.
Statistical Comparison of Body Weights and of Metabolic Rates.

Age group in days	Avg wt of controls g	Avg wt of ovari- ectomized g	Significance ratio	No. of con- trol rats	No. of ovariec- tomized rats	Controls cal*	Ovariecto- mized cal*	Signifi- cance ratio
40-60	80	85	1.2956	12	16	827	830	0.6890
90-105	142	163	4.5450	20	18	849	950	6.7428
175-210	177	195	1.6367	32	40	706	697	0.5100
800-840	220	221	0.0791	6	9	773	776	0.0876

* Calories per square meter of skin surface per 24 hours.

creased as the animals became older. The controls reached their maximum average weight about the 55th week which was followed by a gradual decrease. The difference in the average weights of the two groups became progressively less as the animals became older.

With respect to the changes in the metabolic rate (Table I) the difference was not significant in the 40-60 day group, definitely significant in the 90-105 day group, and not significant in the 175-210 or the 800-840 day groups.

It might be mentioned that a few metabolic determinations were made on these animals when they were around 150 days of age (results not included in above data), and, judging from the results of these few determinations, the mean heat production was about 50 calories per square meter of skin surface per 24 hr lower in the ovariectomized animals than in the controls. The author wonders if this is not the age when the deposited fat is greatest in the test animals and also possibly the time when those factors which are responsible for the accelerated rate of growth and metabolism in the 90-105 day group are beginning to exert less effect upon bodily functions. This point would bear further investigation.

It is interesting to note that the metabolism of the 800-840 day group was somewhat higher than in the 175-210 day group. This confirms the observation of Black,¹³ who reported a higher metabolic rate in 700 day old rats than

in those 7-8 months old. Benedict and MacLeod,¹⁴ also report that metabolism increases with an increase in age in the rat.

The complex relationship between the ovarian hormones and other hormones associated with growth and metabolism make it inadvisable to speculate as to the basic causes of the changes herein reported, but the work does point to a variety of experiments which may be designed to help improve our understanding of the basic phenomena noted. Studies on the effect of combined hypophysectomy and ovariectomy or combined thyroidectomy and ovariectomy are possible approaches, especially if combined with injections of pituitary or thyroid hormones with parallel studies on basal metabolism.

Conclusions. Following the removal of the ovaries in young rats (26th day of age) the animals will gain weight much faster than the control rats for the first few weeks and will remain heavier at least up to 800 days of age. During the growing period when the rate of growth of the ovariectomized rats is significantly greater than the controls the metabolic rate is significantly higher in the former than the latter. The metabolic rate of the normal and ovariectomized rat will be higher at 800 days of age than at 200 days of age, but less at either of these ages than at 90-105 days when growth is taking place most rapidly.

¹⁴ Benedict, F. G., and MacLeod, Grace, *J. Nut.*, 1929, **1**, 367.

¹³ Black, A., *J. Nut.*, 1939, **17**, 361.

Studies of Listerellosis. VI. Isolation of *Listerella monocytogenes* from Liver of Pig.

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In 15 natural outbreaks of ovine and bovine listerellosis in Illinois¹ including 7 in sheep and 8 in cattle, characterized by encephalitic syndrome, *Listerella monocytogenes* was not encountered in the tissues of affected animals other than the central nervous system. In one instance *Listerella monocytogenes* was isolated from the spleen of a heavily parasitized chicken² showing clinical symptoms of coryza. A heavy mortality had occurred in this flock but the primary nature of the disease as well as the cause of the loss was not established. In another instance, *Listerella monocytogenes* was isolated from the stomach contents of an aborted bovine fetus.³ This strain apparently induced abortion following intravenous injection of an experimental pregnant heifer.⁴ The present report concerns the isolation of *Listerella monocytogenes* from the liver of a pig (specimen G89876). This animal did not display clinical symptoms indicative of a central nervous system involvement and represents the third occasion in this laboratory in which *Listerella* has been isolated from tissue other than the central nervous system.

The pig yielding *Listerella monocytogenes* was one of a litter raised on a stock farm in central Illinois and at the time of examination weighed approximately 30 pounds. According to the owner, this animal had exhibited symptoms of intermittent diarrhea and failed to gain in weight as compared to litter mates. On arrival at the laboratory the animal appeared listless and dull but history and clinical symp-

toms failed to suggest the nature of the illness. All other animals in the herd were apparently healthy.

Postmortem examination of the pig failed to reveal gross pathological changes other than a small number of grayish foci of match-head size on the surface of the liver. The character of these foci was not determined. The heart blood, liver, and spleen were cultured on blood agar and MacConkey's agar plates. Following 48 hours incubation at 37°C, the liver blood agar plate cultures yielded pure and abundant growth of a Gram positive rod, possessing biochemical characters indistinguishable from *Listerella*. Fermentation reactions in differential sugars including dextrose, lactose, maltose, sucrose, arabinose, rhamnose, xylose, galactose, levulose, trehalose, raffinose, inulin, dulcitol, mannitol, sorbitol, inositol, salicin, and changes in litmus milk were similar to those of 2 *Listerella* strains, isolated from the central nervous system of naturally affected sheep.

Unilateral supraconjunctival inoculation of the culture into three guinea pigs produced purulent conjunctivitis and keratitis within 4 days. One of 2 rabbits exposed in a similar manner proved resistant. *Listerella monocytogenes* was isolated from the conjunctiva of the 3 experimentally exposed guinea pigs. Agglutination of a suspension of the *Listerella monocytogenes* isolated from the pig liver (G89876) occurred in a dilution of 1:50 in tube antigen and in approximately 1:200 in the rapid plate test using a *Listerella* immune equine serum. There was no agglutination with normal bovine serums in 1:12.5 in the tube and 1:25 on the plate.

Evidence suggesting the pathogenesis of *Listerella monocytogenes* for swine was previously noted following experimental exposure of healthy pigs but the natural disease accompanied by encephalitic symptoms had not been observed in swine in Illinois. However, Biester

¹ Graham, Robert, Levine, N. D., and Morrill, C. C., *Bull.* 499, University of Illinois Agricultural Experiment Station.

² Hurt, H. R., Levine, N. D., and Graham, Robert, *Am. J. Vet. Res.*, 1941, **2**, 279.

³ Graham, Robert, Hester, H. R., and Levine, N. D., *Science*, 1939, **90**, 336.

⁴ Graham, Robert, Hester, H. R., and Levine, N. D., *J. Infect. Dis.* 1940A, **66**, 91.

and Schwarte⁵ reported natural outbreaks of *Listerellosis* in swine and the isolation of *Listerella monocytogenes* from the central nervous system of naturally affected pigs showing clinical symptoms of a central nervous system disorder including incoordination,

⁵ Biester, H. E., and Schwarte, L. H., *Am. J. Vet. Med. Assn.*, 1940, **92**, 339.

stilted gait, trembling, and paralysis of posterior extremities.

Summary. Isolation of *Listerella monocytogenes* from the liver of a pig unaccompanied by encephalitic symptoms is described. The presence of *Listerella monocytogenes* in the liver raises the question of its pathologic significance in non-encephalitic syndromes swine.

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Biological Activity of Synthetic d,l-Desthiobiotin.

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Optically active desthiobiotin was first obtained by du Vigneaud, Folkers and coworkers^{1,2} as a degradation product of natural biotin. The Cornell group made the interesting observations that desthiobiotin is equally as effective as biotin in stimulating the growth of *Saccharomyces cerevisiae*,² but has an anti-biotin effect for *Lactobacillus casei*. They further provided evidence³ to show that the growth effect on the yeast is probably due to the conversion of desthiobiotin to biotin by the yeast cell. Lilly and Leonian⁴ and Stokes and Gunness⁵ have demonstrated the ability of desthiobiotin to replace biotin in the nutrition of various microorganisms.

Biological data on the activity of desthiobiotin for rats have, however, been lacking. In a recent report on the biological activity of synthetic biotin and several intermediates, Emerson⁶ remarked that "desthiobiotin shows a low order of activity in biotin-depleted rats.

However, the response was irregular and the slight activity of this compound may have been due to the presence of traces of biotin, since the compound was prepared from biotin." Since synthetic d,l-desthiobiotin⁷ known to be free of biotin, has been available to us for some time, we have examined its activity for microorganisms and biotin-deficient rats in some detail.*

Experimental. The various samples of d,l-desthiobiotin tested melted at 163-164°C (corr.). The substance was dissolved for the assays in one of two ways: (1) When dilute solutions were required, as for the microbiological experiments or for some of the bioassays, it was dissolved in water, in which the solubility is about 0.1%, giving a solution of pH 3.5; (2) when more concentrated solutions were needed, as in bioassay dosage at higher levels, the compound was dissolved in somewhat less than the stoichiometric amount of sodium hydroxide solution to give a pH of 6-7.

Microbiological assays. The effect of d,l-desthiobiotin on the growth of *Saccharomyces cerevisiae* No. 139 was examined with 2 different media, that of Snell, Eakin and

¹ du Vigneaud, V., Melville, D. B., Folkers, K., Wolf, D. E., Mozingo, R., Keresztesy, J. C., and Harris, S. A., *J. Biol. Chem.*, 1942, **146**, 475.

² Melville, D. B., Dittmer, K., Brown, G. W., and du Vigneaud, V., *Science*, 1943, **98**, 497.

³ Dittmer, K., Melville, D. B., and du Vigneaud, V., *Science*, 1944, **99**, 203.

⁴ Lilly, V. G., and Leonian, L. H., *Science*, 1944, **99**, 205.

⁵ Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, 1945, **157**, 121.

⁶ Emerson, G. A., *J. Biol. Chem.*, 1945, **157**, 127.

⁷ Duschinsky, R., and Dolan, L. A., to be published.

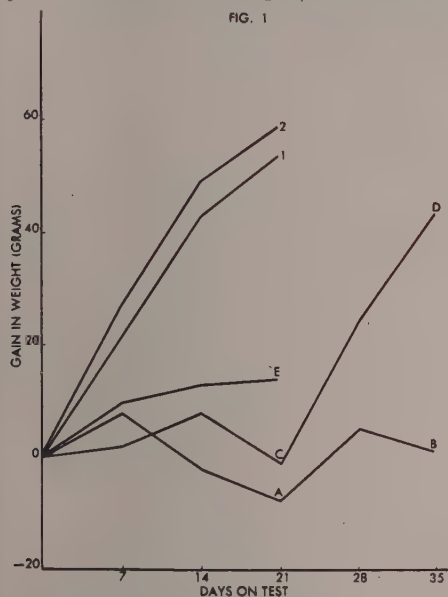
* The samples of d,l-desthiobiotin were made available to us by Dr. R. Duschinsky.

⁸ Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 175.

Williams⁸ and that of Hertz.⁹ With both media, half-maximum growth was obtained with about 0.03 millimicrograms of biotin per cc of medium; the Hertz medium supported better growth. *d,l*-Desthiobiotin showed an average biotin activity of 53% by weight in the medium of Snell *et al.* and 50% in the Hertz medium. These values are in essential agreement with the values reported by Wood and du Vigneaud¹⁰ for synthetic *d,l*-desthiobiotin and by Harris *et al.*¹¹ for *d,l*-desthiobiotin obtained from *d,l*-biotin.

The "molar inhibition ratio" of *d,l*-desthiobiotin was determined in the manner described by Dittmer and du Vigneaud¹² with the

FIG. 1



Effect of Desthiobiotin with Diet No. 1 (30% egg white).

Curve 1 : 0.5 µg of biotin

2 : 1 µg of biotin

O-A: Negative controls; A-B: 1 mg desthiobiotin; O-C: 1 mg desthiobiotin; C-D: 10 mg desthiobiotin; O-E: 0.5 mg desthiobiotin.

All given by intraperitoneal injection.

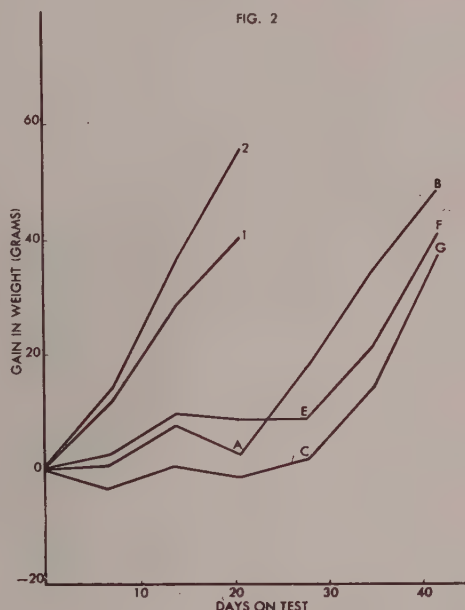
⁹ Hertz, R., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 15.

¹⁰ Wood, J. L., and du Vigneaud, V., *J. Am. Chem. Soc.*, 1945, **67**, 210.

¹¹ Harris, S. A., Mozingo, R., Wolf, D. E., Wilson, A. N., Arth, G. E., and Folkers, K., *J. Am. Chem. Soc.*, 1944, **66**, 1800.

¹² Dittmer, K., and du Vigneaud, V., *Science*, 1944, **100**, 129.

FIG. 2



Effect of desthiobiotin with Diet No. 2 (10% egg white).

Curve 1 : 0.5 µg of biotin

2 : 1 µg of biotin

O-A: Negative controls; A-B: 1 mg desthiobiotin; O-C: 4 µg desthiobiotin; O-G: 1 mg desthiobiotin; O-E: 8 µg desthiobiotin; E-F: 1 mg desthiobiotin.

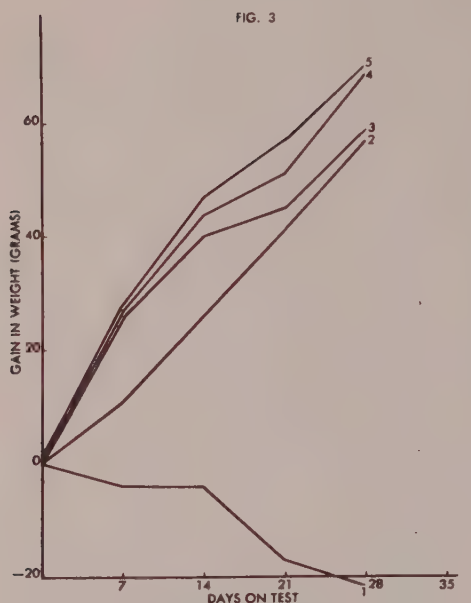
All administration was by intraperitoneal injection except C-G where administration was oral.

L. casei method of Shull, Hutchings and Peterson,¹³ modified as described by Shull and Peterson.¹⁴ The average value of the "molar inhibition ratio" found in 3-day runs was 17,000; Dittmer and du Vigneaud¹² reported 9,100 for *d*-desthiobiotin. Since our value for *d,l*-desthiobiotin is approximately twice the value for *d*-desthiobiotin, the inference is that *l*-desthiobiotin does not act as an antibiotin. This conclusion cannot be drawn with certainty, however, because of the somewhat empirical nature of the test for inhibition. The final decision must await simultaneous runs with the pure optical isomers.

The "molar inhibition ratio" is usually determined with a 3-day incubation period in accord with the usual assay practices. When

¹³ Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

¹⁴ Shull, G. M., and Peterson, W. H., *J. Biol. Chem.*, 1943, **151**, 201.



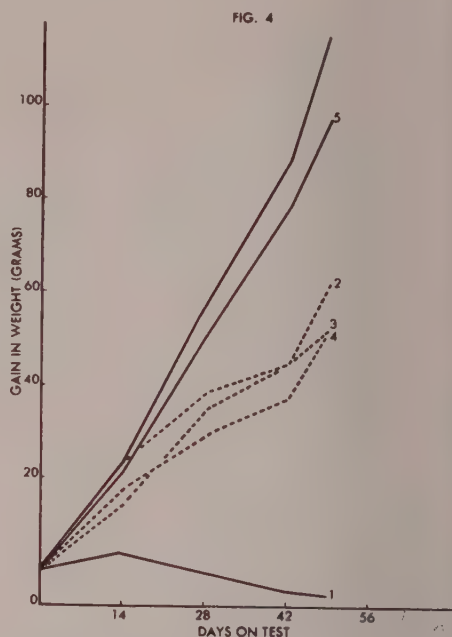
Effect of desthiobiotin with Diet 1 (30% egg white).

- Curve 1 : Negative controls
 2 : 0.35 µg biotin
 3 : 4 mg desthiobiotin
 4 : 0.7 µg biotin
 5 : 8 mg desthiobiotin.

All administered by intraperitoneal injection.

the time of incubation was extended to 6 days, it was found that the value of the ratio increased somewhat, indicating a small diminution in antibiotin activity. The increases observed, however, fell within the limits of variation of random runs and hence were not necessarily significant.

Biological assays. Two procedures can be used for inducing biotin deficiency in the rat, *viz.*, by the avidin mechanism or by repression of intestinal synthesis of biotin by means of sulfonamide compounds. Both approaches have been used in the present experiments. Because of the dearth of information regarding the quantitative aspects of biotin assay with diets containing egg white, it seemed desirable to vary the content of egg white. Accordingly, diet No. 1 (Table I) is patterned after that employed by Dittmer, du Vigneaud, György and Rose¹⁵ and contains 30% dried egg white as the source of avidin and as the sole source of protein; diet No. 2



Effect of desthiobiotin with Diet No. 3 (Succinylsulfathiazole).

- Curve 1 : Negative controls
 2 : 3000 units folic acid concentrate
 3 : 0.4 µg of desthiobiotin + 3000 units of folic acid
 4 : 0.8 µg of desthiobiotin + 3000 units folic acid
 5 : 1 mg of desthiobiotin + 3000 units folic acid
 6 : 0.2 µg of biotin + 3000 units folic acid.

All administered orally. The broken lines indicate groups which showed marked biotin deficiency symptoms at the end of 7 weeks.

is based upon that used by Nielsen and Elvehjem¹⁶ and provides 10% egg white and 8% casein. For the sulfonamide experiments, 1% succinylsulfathiazole was included in a diet similar to that employed by Daft and Sebrell¹⁷ in their experiments on folic acid. The diets are detailed in Table I.

25-day-old male rats (Sprague-Dawley), weighing 35-45 g, were housed in individual

¹⁵ Dittmer, K., du Vigneaud, V., György, P., and Rose, C. S., *Arch. Biochem.*, 1944, **4**, 229.

¹⁶ Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **141**, 405.

¹⁷ Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1943, **58**, 1542.

TABLE I.
Composition of the Diets.
(g per 100 g of diet)

	115	216	317
Sucrose		73	
Glucose (Cerelease)			72
Corn starch	48		
Dried egg white	30	10	
Vitamin-free casein (Labco)		8	18
Salts (USP No. 1)	5	4	4
Wesson oil	14		3
Primex		5	
Cod liver oil	3		2
Succinylsulfathiazole			1

The following supplement was supplied to the rats receiving diets 1 and 2 three times weekly in 1 ml of distilled water: 47 μ g of thiamine chloride, riboflavin and pyridoxine, 470 μ g of calcium pantothenate, 1.17 mg of inositol and nicotinic acid, 580 μ g of p-aminobenzoic acid, and 7 mg of choline chloride. In addition the rats on diet 2 received 0.2 ml of USP Cod Liver Oil weekly.

The rats on diet 3 received the following supplement: 233 μ g of thiamine chloride and pyridoxine, 470 μ g of riboflavin and calcium pantothenate, 1.17 mg of inositol, 580 μ g of p-aminobenzoic acid, 187 μ g vitamin K (Synkavite), 2.33 mg nicotinic acid, 23.3 mg choline chloride, 3 times weekly in 4 ml of water.

cages and fed these diets *ad libitum*. Each curve in the figures represents the average response of a group of 3 to 5 rats. All supplements are given in terms of daily dosages.

Activity of *d,l*-Desthiobiotin in Egg White Experiments. After an average period of 45 days on diet 1 (30% egg white) and 65 days on diet 2 (10% egg white), the rats showed marked symptoms of biotin deficiency, characterized by cessation of growth, spectacled eye, seborrheid dermatitis and alopecia; arched back and profuse salivation were also manifested by many of the rats. Supplementation with biotin or *d,l*-desthiobiotin was begun at the times stated above.

Preliminary experiments with both diets showed that *d,l*-desthiobiotin, administered either orally or intraperitoneally in daily doses up to 8 μ g, had less than 5% of the activity of biotin. However, subsequent experiments with larger doses revealed that definite biotin activities of the order of magnitude of 0.1-0.01% could be elicited. Thus, rats that had shown no weight gain on diet 2 (10% egg white) when given 0, 4 or 8 μ g per day of *d,l*-desthiobiotin (Fig. 2: O-A, O-C, O-E), responded with marked weight gains (Fig. 2: A-B, C-G,

E-F) and amelioration of the deficiency symptoms when dosed with 1 mg of the compound (A-B and E-F intraperitoneally, C-G orally). When, however, 1 mg was administered intraperitoneally to rats on diet 1 (30% egg white), no such response resulted (Fig. 1: O-C, A-B); 10 mg was required to cause a marked weight response (Fig. 1: C-D) and cure of symptoms. The experiment with diet 1 was repeated (Fig. 3) with essentially the same result. It appears, therefore, that the small biotin activity of *d,l*-desthiobiotin was inversely proportional to the concentration of egg white in the diet.

This inverse effect suggests that desthiobiotin may have acted in these experiments by displacement of biotin from avidin-biotin complex. Dittmer and du Vigneaud¹² have shown that desthiobiotin combines with avidin and that two analogs of desthiobiotin, namely, 4-(imidazolidone-2)-caproic acid and 4-(imidazolidone-2)-valeric acid, can displace biotin from avidin-biotin as tested by yeast or *L. casei* cultures. The latter has not been demonstrated for desthiobiotin because of the experimental difficulties imposed by the behavior of desthiobiotin towards these microorganisms. The present animal experiments provide some evidence for this viewpoint.

Activity of *d,l*-Desthiobiotin in Experiments with Succinylsulfathiazole. The results were similar to those obtained in the egg white experiments in that the apparent activity was of a low order of magnitude, approximately 0.02% of biotin. Thus, as shown in Fig. 4, 1 mg of *d,l*-desthiobiotin was required to prevent deficiency symptoms and effect a weight increase similar to that obtained with 0.2 μ g of biotin.

These experiments were complicated by folic acid deficiency as demonstrated by depression of the leucocyte count. After 4 weeks on the basal diet, the rats had reached stationary weight, but did not show clean-cut symptoms of biotin deficiency. At that point, supplementation with the biotin compounds and a folic acid concentrate was begun. The latter was treated with 0.3% hydrogen peroxide for 24 hr at room temperature to render the biotin present unavailable to the rat.¹⁸ Microbiological assay with *S. lactis*¹⁹ showed that

the folic acid was not affected by the peroxide treatment. Daily supplementation with 3000 Snell-Peterson units²⁰ of folic acid *precipitated marked symptoms of biotin deficiency* in groups 2, 3 and 4 (Fig. 4). The negative controls, group 1, which received neither biotin nor folic acid, showed only slight symptoms of biotin deficiency (spectacled eye). Group 5 and 6, which received 1 mg of d,1-desthiobiotin and 0.2 µg of biotin respectively, showed no manifestations of biotin deficiency. This must therefore be considered as a prophylactic experiment. The apparent biotin activity due to d,1-desthiobiotin was of the order of magnitude of 0.02%.

The striking effect of the folic acid concentrate in accentuating the biotin deficiency symptoms may arise from the fact that folic acid induces better growth and survival and thus increases the biotin requirement of the rats. Similar observations have been recorded by Daft, Ashburn and Sebrell,²¹ who produced

a dermatitis in rats by supplementing a diet containing succinylsulfathiazole with a liver concentrate which provided folic acid, among other things; in "the 40 rats given neither biotin nor liver extract, . . . any degree of biotin deficiency present in these animals was difficult to diagnose." There are indications in the recent report of Nielsen and Black²² that the same is true for mice.

Summary. Synthetic d,1-desthiobiotin induces the following biological responses. (1) It is 50% as active as d-biotin in promoting the growth of *S. cerevisiae*. (2) It is approximately half as effective as d-desthiobiotin in inhibiting fermentation by *L. casei*. The inhibitory effect may diminish slightly over a period of 6 days. (3) On diets containing egg white or succinylsulfathiazole, the apparent vitamin activity in rats is of a low order of magnitude, 0.1 to 0.01% that of biotin.

²¹ Daft, F. S., Ashburn, L. L., and Sebrell, W. H., *Science*, 1942, **96**, 321.

²² Nielsen, E., and Black, A., *J. Nutrition*, 1944, **28**, 203.

The assistance of Miss Gertrude Engel with some of the microbiological assays is gratefully acknowledged.

¹⁸ Nielsen, E., Shull, G. M., and Peterson, W. H., *J. Nutrition*, 1942, **24**, 523.

¹⁹ Luckey, T. D., Briggs, G. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

²⁰ Snell, E. E., and Peterson, W. H., *J. Bact.*, 1940, **39**, 173.

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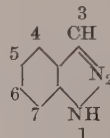
The In vitro Antibacterial Effects of Sulfanilamidoindazoles.

C. A. LAWRENCE AND G. R. GOETCHIUS. (Introduced by M. L. Tainter.)

From the Research Laboratories of Winthrop Chemical Company, Inc., Rensselaer, N. Y.

In a previous report from these laboratories, Kwartler and Lucas¹ described the preparation of several sulfanilamidoindazoles. This paper presents the results of the bacteriological investigations on 4 of the compounds, 3-, 5-, 6-, and 7-sulfanilamidoindazole. To our knowledge the only reference to the use of compounds of this nature in chemotherapy is the

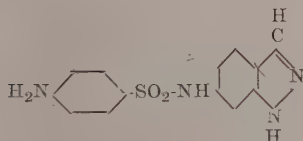
work of Coggeshall and Maier² who found that 5-sulfanilamidoindazole is ineffective in experimentally induced influenza and poliomyelitis infections in mice. The structural formula for indazoles, together with the system for numbering, is as follows:



¹ Kwartler, C., and Lucas, P., *J. Am. Chem. Soc.*, 1943, **65**, 1804.

² Coggeshall, L. T., and Maier, J., *J. Pharm. and Exp. Therap.*, 1942, **76**, 161.

The compounds which were used in these studies had a sulfanilamide radical substituted in either the 3-, 5-, 6-, or 7-position; *e. g.*, 6-sulfanilamidoindazole has the following structure.



Methods and Results. Inasmuch as these compounds are comparatively insoluble in water, they were used in the form of their sodium salts. A series of dilutions of each compound, varying from 1:100 to 1:10,000, was prepared in distilled water. To tubes containing 4.4 cc of nutrient medium was added 0.5 cc of the aqueous drug solution. The tubes were autoclaved at 10 lb for 10 min, and, upon cooling, each tube was inoculated with 0.1 cc of a 1:200 broth dilution of a 24-hr culture of test organism. The final drug concentrations were, therefore, 1/10 the original concentrations of the aqueous solutions. Veal infusion broth containing 0.15% dextrose and 0.1% horse serum was used in testing the compounds against the pneumococcus and streptococcus. The *Brucella* organisms were tested in tryptose

phosphate broth containing 0.1% agar. Beef extract broth was used for *Staphylococcus aureus* and several gram-negative enteric organisms. Studies on the anaerobic *Clostridia* were carried out in Brewer's fluid thioglycollate medium.

The inoculated test media, with controls, were incubated at 37°C and observed for visible growth after 24, 48, and 72 hr. Due to their slower rate of growth, the *Brucella* were observed at 48, 72, and 96 hr. The test compounds were considered to show bacteriostatic action when the tubes containing them showed less than one-half the growth noted in the broth controls at the end of the initial incubation period. In order to differentiate between bacteriostatic and possible bactericidal effects, lack of visible growth in the drug-broth tubes at the end of 72 hr (or 96 hr for the *Brucella*) was checked for the presence of viable organisms by transferring three 4-mm loopfuls of the test medium to a tube containing the same medium without the drug. Absence of growth in the subculture tubes indicated that the organisms were destroyed in the original medication tubes.

The results of tests against the several bacteria are presented in Table I. Although not indicated in the table, the sulfanilamidoindazoles compare with sulfathiazole and sulfadia-

TABLE I.
Highest Dilution of Four Sulfanilamidoindazoles Exhibiting Antibacterial Action.

Organism	Sulfanilamidoindazole							
	3-		5-		6-		7-	
	Bs × 1000	Bc × 1000	Bs × 1000	Bc × 1000	Bs × 1000	Bc × 1000	Bs × 1000	Bc × 1000
<i>Pneumococcus</i> Type I	5	5	10	10	10	10	25	25
" " II	10	10	10	5	10	5	20	15
" " III	5	5	5	5	10	5	20	15
<i>Strep. hemolyticus</i>	<5	<5	5	<5	5	<5	<5	<5
<i>Staph. aureus</i>	2.5	<1	5	<1	40	<1	50	<1
<i>E. typhi</i>	2.5	<1	7.5	<1	5	<1	5	<1
<i>S. paratyphi A</i>	2.5	<1	5	<1	2.5	<1	<1	<1
<i>S. dysenteriae</i>	2.5	<1	5	<1	2.5	<1	1	<1
<i>Prot. vulgaris</i>	5	<1	7.5	<1	2.5	<1	50	<1
<i>V. cholerae</i>	50	1	50	<1	50	1	50	<1
<i>Br. abortus</i>	100	10	25	5	100	10	100	10
" <i>melitensis</i>	100	10	25	7.5	100	10	100	10
" <i>suus</i>	100	2.5	75	2.5	100	2.5	100	5
<i>Cl. welchii</i>	<1	<1	<1	<1	<1	<1	<1	<1
" <i>tetani</i>	<1	<1	<1	<1	<1	<1	<1	<1
" <i>histolyticus</i>	<1	<1	<1	<1	<1	<1	<1	<1
" <i>oedematiens</i>	<1	<1	<1	<1	<1	<1	<1	<1

Bs = Bacteriostatic

Bc = Bactericidal

< = Greater concentrations not tested.

TABLE II.
Comparison of the Effects of Sulfathiazole and 6-Sulfanilamidoindazole Upon *Brucella melitensis* in the Presence of Paba.

Paba	6-Sulfanilamidoindazole						Sulfathiazole			
	1:16,000		1:32,000		1:64,000		1:1000		1:2000	
	24	48	24	48	24	48	24	48	24	48 hr
1:1000	0	0	0	1	1	4	1	2	2	4
1:5000	0	0	2	2	2	4	4	4	4	4
1:10,000	0	0	1	3	3	4	4	4	4	4
1:50,000	0	0	1	3	3	4	4	4	4	4
1:100,000	0	0	1	3	1	4	4	4	4	4
1:500,000	0	0	1	3	1	4	4	4	4	4
None	0	0	1	1	1	1	1	2	2	2

0 = No growth (bacteriostatic only)

1-3 = Slight to heavy growth

4 = Maximum growth

zine in degree of activity against most of the bacteria. The *Brucella* organisms, however, are more sensitive to the sulfanilamidoindazoles than to sulfathiazole or sulfadiazine. Under corresponding experimental conditions, the latter compounds were bacteriostatic in dilutions of 1:16,000 to 1:32,000 but failed to exhibit a bactericidal action against the *Brucella* even when used in concentrations of 0.1%

Tests with p-Aminobenzoic Acid. While studies on *p*-aminobenzoic acid-sulfonamide relationships were being conducted, it was noted that the sulfanilamidoindazoles are relatively resistant to antagonism by *p*-aminobenzoic acid. A series of tests was consequently carried out to measure the degree of antagonism displayed by *p*-aminobenzoic acid (PABA) against the antibacterial activity of the sulfanilamidoindazoles and to compare it to the extent of antagonism shown by PABA against sulfathiazole. Dilutions of 1:100, 1:500, 1:1000, 1:5000, 1:10,000, and 1:50,000 of PABA were prepared in water. Of each dilution of the acid 0.5 cc was added to 4.5 cc of tryptose-phosphate-0.1% agar broth containing either a 1:16,000, 1:32,000, or 1:64,000 dilution of the sodium salt of one of the sulfanilamidoindazoles. Sodium sulfathiazole was used in dilutions of 1:1000 and

1:2000. After autoclaving, each tube was inoculated with a loopful of a 24-hr broth culture of *Brucella melitensis*. The amount of visible growth developing in the tubes was observed after 24 and 48 hr of incubation at 37°C. Table II presents the results of a typical test using 6-sulfanilamidoindazole. In general, results comparable to those obtained with this compound were also noted when 3-, 5-, and 7-sulfanilamidoindazoles were used.

It may be readily observed (Table II) that 6-sulfanilamidoindazole is only slightly affected by the presence of PABA. This effect is noticeable only in the zone where the end-point of the bacteriostatic activity of the compound occurs (1:32,000-1:64,000). Unlike 6-sulfanilamidoindazole, sulfathiazole in high concentration loses its bacteriostatic activity in the presence of corresponding amounts of PABA.

Summary. The *in vitro* antibacterial effects of 3-, 5-, 6-, and 7-sulfanilamidoindazole against a variety of bacteria are presented. Data are also given to show that there is a several-thousand-fold decrease in the antagonism displayed by *p*-aminobenzoic acid against the sulfanilamidoindazoles as compared with its antagonism against sulfathiazole.

The authors wish to acknowledge the technical assistance of Virginia L. Wilson.

The Interrelation of Corresponding Complement Components of Man and Guinea Pig.*

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A question of considerable interest is whether or not the corresponding components of human and guinea-pig complements are interchangeable or "mutually substitutive." The identity of corresponding components of the two species has been an assumption in at least two important studies on complement by Hegedüs and Greiner¹ and Heidelberger and Mayer,² but Ecker, Pillemer, and Seifter,³ reported that: "(1) Human C'1 can replace guinea-pig C'1, but guinea-pig C'1 cannot effectively replace human C'1. (2) Human C'2 cannot effectively replace guinea-pig C'2, but guinea-pig C'2 can replace human C'2. (3) Human C'3 can replace guinea-pig C'3, and guinea-pig C'3 can replace human C'3. (4) Human C'4 can replace guinea-pig C'4, but guinea-pig C'4 cannot replace human C'4."

In these conclusions, drawn from experiments with specifically inactivated complements of both species, the word "effectively" was used as a modifier, because the reasons for the failure of replacement were not understood, and it was thought that anti-complementary effects of the testing fractions (specifically inactivated complements) might be implicated. In a dissertation from this laboratory presented in May, 1944⁴ it was pointed out that, "in light of data accumulated in the present research of the effective concentrations of the components of human serum, the experiments

on which the above conclusions were based may be re-examined, more reliable conclusions drawn, and further experiments suggested." From the study of the effective concentrations of the individual complement components, it was concluded that the amounts of certain components used in the original experiments were in some cases too small, particularly in those instances in which effective substitution of the corresponding components was not achieved. Accordingly, another series of experiments, using larger amounts of the indicated components, was conducted. In addition, the availability of more highly purified complement fractions made it possible to attack the problem more directly than had been true previously. A series of experiments was then conducted by use of single components of the complement of one species with the specifically inactivated complements of the other species.

Methods. Specifically inactivated guinea-pig complements were prepared by the methods summarized in the review by Ecker and Pillemer.⁵ Specifically inactivated human complements were prepared, as described by Ecker, Pillemer, and Seifter.³ Purified fractions of guinea-pig complement were prepared by ammonium sulfate fractionation, according to the methods of Pillemer, Ecker, Oncley, and Cohn⁶; and of those of human complement by the methods summarized by Ecker, Seifter, and Dozois.⁷

Complement was titrated by the method of complete hemolysis,³ a unit of complement (or the unit equivalent volume of a specifically inactivated complement) being defined as the

* Aided by a grant from the Commonwealth Fund.

¹ Hegedüs, A., and Greiner, H., *Z. Immunitätsforsch.*, 1938, **92**, 1.

² Heidelberger, M., and Mayer, M., *J. Exp. Med.*, 1942, **75**, 285.

³ Ecker, E. E., Pillemer, L., and Seifter, S., *J. Immunol.*, 1943, **47**, 181.

⁴ Seifter, S., Dissertation, Western Reserve University, 1944.

⁵ Ecker, E. E., and Pillemer, L., *Ann. N. Y. Acad. Sc.*, 1942, **43**, 63.

⁶ Pillemer, L., Ecker, E. E., Oncley, J. L., and Cohn, E. J., *J. Exp. Med.*, 1941, **74**, 297.

⁷ Ecker, E. E., Seifter, S., and Dozois, T. F., *J. Lab. and Clin. Med.*, 1945, **30**, 39.

TABLE I.
The Mutual Substitution of Corresponding Components of Human and Guinea-pig Complements
as Demonstrated with Specifically Inactivated Complements.

C'1	Guinea-pig			Human				% hemolysis
	C'2	C'3	C'4	C'1	C'2	C'3	C'4	
	X	X	X	X	X		X	100
X		X	X		X	X	X	55*
X	X		X	X		X	X	95
X	X	X		X		X	X	80
X	X		X		X	X	X	75*
X	X		X	X		X	X	95
		X	X	X	X		X	100
	X	X	X	X	X	X		90†

* Six units of specifically inactivated human complement used.

† Two units of specifically inactivated guinea-pig complement used.

X indicates the presence of the component designated in the column heading.

TABLE II.
The Substitution of Purified Fractions of Human and Guinea-pig Complements.

C'1	Guinea-pig			Human				% hemolysis
	C'2	C'3	C'4	C'1	C'2	C'3	C'4	
	X	X	X	X				95
X		X	X		X			55
X	X	X					X	100
X					X	X	X	55
	X	X		X		X	X	100
	X	X		X	X		X	80

minimal amount of normal serum required to hemolyze completely the test dose of sensitized sheep red blood cells.

The Interrelation of Specifically Inactivated Complements of Man and Guinea-pig. Table I shows the degree of hemolysis of the standard dose of sensitized sheep red cells obtained by the action of various mixtures of specifically inactivated complements of man and guinea-pig. Unless otherwise noted, single unit amounts, as defined above, were used. The table is so constructed that the results may be conceived of as guinea-pig components substituting in a human complement system or *vice versa*. In each case, if sufficient amounts of the specifically inactivated complements were used, a significant degree of hemolysis was obtained, demonstrating the capacity of the corresponding components of one system to substitute effectively in the other system. Results not shown in the table again demonstrated that only corresponding components can replace one another; that is, a mixture of complements, similarly inactivated, of the two species produced no hemolysis of the sheep cells.

The Substitution of Purified Complement

Fractions. Table II shows the degree of hemolysis obtained with the use of various mixtures of the specifically inactivated complements of man or guinea-pig and the available purified fractions of guinea-pig or human complement. The guinea-pig fraction containing C'2 also had some C'3 activity, and so was used to test the capacity of these components for completing human systems lacking C'2 and C'3 respectively. Again the results show that corresponding components of the two systems are "mutually substitutive."

Summary and Conclusions. Under the proper conditions of concentration, all of the corresponding complement components of man and guinea-pig are mutually substitutive. This represents a revision of the view previously expressed³ that only certain substitutions are effective. Nevertheless, it is to be borne in mind that in those cases in which specifically inactivated complements of the two species are used together, the full effect of component substitution may be inhibited or concealed. Such an aberration may be due to (a) the increased concentration of anti-complementary substances in these fractions, particularly in the globulin concentrates, or (b) the disarrange-

ment of the complement complex in the course of specific inactivation by the recommended procedures.

It is pertinent to report that the corresponding components of rabbit and human complements have also been found to be mutually substitutive.⁸

Since submitting this article we have become acquainted with an excellent paper by O. G. Bier, G. Leyton, M. M. Mayer, and M. Heidelberger

⁸ Dozois, T. F., Seifter, S., and Ecker, E. E., in preparation.

which appears in the May, 1945, issue of the *J. Exp. Med.* These authors demonstrate that corresponding components of guinea pig and human complements are mutually substitutive; and emphasize that testing reagents for complement components must contain an excess of the desired components and must be used in dilutions which ensure absence of anti-complementary effects. Further, though it came to our attention too late for incorporation in the present paper, the suggestion of Bier *et al.* that C' component titers be expressed solely in units is constructive, and will be observed in our subsequent publications.

14955 P

Rapid and Submerged Growth of Mycobacteria in Liquid Media.

RENE J. DUBOS.

From the Rockefeller Institute for Medical Research, New York City.

Attempts to obtain rapid and diffuse growth of virulent tubercle bacilli have led us to recognize that certain complex lipids exert a remarkable stimulatory effect on the multiplication of mycobacteria. Particularly striking results have been obtained with the following materials (a) phosphatide fractions prepared from egg yolk, cattle brain, human erythrocytes and soya bean.* (b) synthetic non-ionic surface active agents consisting of esters of long chain fatty acids and of polyhydric alcohols.†

The growth promoting effect of these substances has been tested with 5 strains of saprophytic mycobacteria, 5 virulent avian strains, 1 bovine strain (Ravenel) and 2 human strains (H37 RV and Jamaica No. 22). The results indicate that the different groups of mycobacteria (saprophytic, avian, bovine, and human) differ greatly in their optimal requirements with reference to the lipids mentioned above; on the other hand, within one given group, the different strains tested have exhibited a striking similarity of behavior. The avian strains are only little affected by the phosphatide preparations but are greatly stimulated by the fatty acid esters; on the contrary the bovine and human strains respond remarkably well

to the different phosphatides but are inhibited by minute concentrations of the non-ionic surface active agents. The following table illustrates the composition of satisfactory media prepared by adding to Long's medium different concentrations of soya bean phosphatide and of a polyoxyalkylene derivative of sorbitan monostearate (Tween 60).‡

Mycobacteria can be made to grow in the lipid media without the necessity of floating

* The brain and red cell phosphatides were prepared by Dr. Jordi Folch-Pi. The soya bean phosphatide was obtained through the courtesy of Dr. A. Scharf of Associated Concentrates, Inc., Atlanta, Georgia.

† A variety of these synthetic non-ionic surface active agents are produced by the Atlas Powder Company, Wilmington, Delaware, and by the Glyco Products Co., Inc., Brooklyn, N. Y. Although the present report deals only with the product marketed under the name of Tween 60 by the former company, similar results have been obtained with a number of other long chain fatty acid esters of polyhydric alcohols.

‡ Tween 60 is directly dispersible in water. The soya bean phosphatide was dissolved in ether and dispersed from ether solution into water. Both these products can be autoclaved with Long's medium.

TABLE I.

Strains of Mycobacteria	Long's medium (2% glycerine or 0.5% glucose). +					
Saprophytic	0	-0.05%	Soya bean phosphatide	+	0.02-0.2	% Tween 60
Avian	0	-0.05%	" "	+	0.02-0.1	% " "
Bovine and Human	0.01-0.05%	" "	" "	+	0	-0.002% " "

the inoculum on the surface of the fluid as is the usual practice with these organisms. The cultures grow readily throughout the fluid and can be transferred repeatedly into the same media. Addition of 0.1 cc of culture to 10 cc of new medium is sufficient to secure growth of the saprophytic and avian strains within 24 hr, and of the bovine and human strains within 72 hr; growth is slower with smaller inocula.

We have also established that the addition of purified serum albumin (0.1% or less) to these liquid media further enhances the growth of tubercle bacilli and permits in particular more rapid multiplication of small inocula.

The cultures obtained under these condi-

tions (with and without serum albumin) are typical in morphology and staining characteristics. When returned to the classical Long's medium (in the absence of the lipid fractions), they manifest again their typical mode of growth, developing slowly, in the form of heaped masses, on the surface of the medium.

Summary. Addition to Long's synthetic medium of small amounts of phosphatides and of long chain fatty acids esters of polyhydric alcohols permits submerged and rapid growth of tubercle bacilli; the different groups of mycobacteria appear to exhibit differential optima with reference to these two types of substances.

14956

The Microtechnical Demonstration of Sites of Lipase Activity.*

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From the Department of Medicine, the University of Chicago.

It is known that lipase is not destroyed by acetone dehydration; in fact, the enzyme is usually prepared from acetone-dried tissues. Nor is it too sensitive to heat; in preliminary experiments no appreciable decrease in activity was observed when acetone-dried pancreas powder was kept at 62°C for 2 hr. On the basis of these facts it appeared feasible to demonstrate lipase in paraffin sections by following the principles of the microtechnical demonstration of phosphatases.¹⁻³ These include the incubation of the slides with an ester,

known to be split by the enzyme, in the presence of a salt the metal ions of which form an insoluble salt with the liberated acid and in this way trap the ions of the acid *in situ*. However, when it was attempted to put these principles into practice, great difficulties were encountered. The substrate must meet three requirements which, for a long time, seemed to be mutually exclusive. First, it must be hydrolyzed by lipase; second, it must be water-soluble since lipase is greatly inhibited even by traces of non-aqueous solvents such as alcohols or ketones;^{4,5} third, it must form insoluble salts with some metal, non-toxic to the enzyme, such as Ca or Mg. Monocarboxylic acids which have water-soluble esters (up to

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

¹ Takamatsu, H., *Tr. Soc. Path. Jap.*, 1939, **29**, 492.

² Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 23.

³ Gomori, G., *Arch. Path.*, 1941, **32**, 189.

⁴ Murray, D. R. P., *Biochem. J.*, 1929, **23**, 292.

⁵ Glick, D., and King, C. G., *J. Biol. Chem.*, 1931, **94**, 497.

C₄) do not have insoluble salts. The esters of those forming insoluble salts (from C₁₂ upward) are insoluble in water. Of the dicarboxylic acids, the water-soluble esters of oxalic acid, the only acid having a highly insoluble Ca salt, are not attacked by lipase.⁶ The salts of the higher dicarboxylic acids which have water-soluble, readily hydrolyzed half-esters (glutaric, adipic, sebacic) are not sufficiently insoluble for the purpose of microtechnical demonstration. A number of attempts with dispersions of water-insoluble esters were uniformly unsuccessful. At this point it was learned that a number of water-soluble long-chain fatty acid esters of polymer glycols and hexitols have recently become available commercially. Samples of several of these compounds were obtained all of which were found to be readily hydrolyzed by a water-glycerol extract of acetone-dehydrated pancreas. A microtechnical method for the demonstration of lipase has been developed by using Tween 40 or Tween 60, palmitic and stearic esters, respectively, of hexitans in which most of the hydroxyl groups are etherified. The Tweens,⁷ manufactured by the Atlas Powder Co., Wilmington, Del., are amber colored, semisolid, unctuous substances which give crystal clear aqueous solutions.

The Tweens were found to be hydrolyzed by pancreatic lipase at a rate about half that of olive oil as determined by the method of Sure, Kik and Buchanan.⁸

The following technic was found to yield uniformly good results:

1. Fix thin slices of fresh tissues in ice cold absolute acetone for 12 to 24 hr. Dehydrate at room temperature in 2 more changes of absolute acetone, 6 to 12 hr each. Embed through 2 changes of benzene, 30 min each, in paraffin. Neither 60°C, nor 2 hr at this temperature should be exceeded.

2. Cut sections around 5 micra. Pass slides through xylene and 2 alcohols to distilled water. Dip slides between the first and second

alcohol into a dilute (0.5%) solution of colodion in alcohol-ether.

3. Incubate slides for 6 to 12 hr at 37°C in the following solution:

2% solution of Tween 40 or 60	5 cc
M/10 maleate buffer pH \pm 7.4,	
30% glycerol	20 " each
2% solution of CaCl ₂	5 "

This solution may be slightly hazy at 37°C. During incubation, the sites of lipase activity will become opaque owing to the precipitation of Ca palmitate or stearate.

4. Rinse slides in distilled water.

5. Transfer to a 2% solution of lead nitrate for 10 min. Ca palmitate or stearate are transformed into the corresponding Pb salts.

6. Rinse slides in many changes of distilled water.

7. Transfer to a dilute solution of yellow ammonium sulfide (about 10 drops to a Coplin jar filled with distilled water) for 2 min. Lead palmitate and stearate will be transformed into dark brown lead sulfide.

8. Wash under the tap. Counterstain with hematoxylin. Dehydrate and mount. It is advisable to use ligroin or dichloroethylene for clearing, and clarite or some similar resin dissolved in the same solvents for mounting, since the stain will gradually fade in media containing xylene.

So far, mainly normal dog and rat organs have been examined by this method. Lipase could be localized in many organs such as liver, pancreas, lung, kidney, testicle, epididymis, adrenal, adipose tissue, stomach and small intestine. In the liver, the largest amount of lipase is found in the central portion of the lobules (Fig. 1). In the pancreas, the site

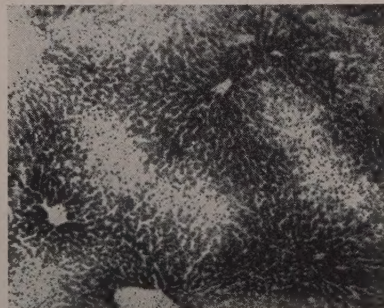


FIG. 1.

⁶ Westenbrink, H. G. K., and Romijn, H. M., *Arch. néerland. de phys.*, 1930, 15, 529.

⁷ Atlas Spans and Atlas Tweens. Atlas Powder Co., 1943.

⁸ Sure, B., Kik, M. C., and Buchanan, K. S., *J. Biol. Chem.*, 1935, **108**, 27.

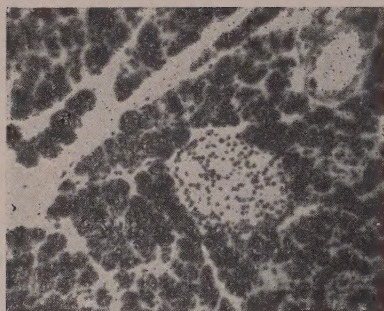


FIG. 2.

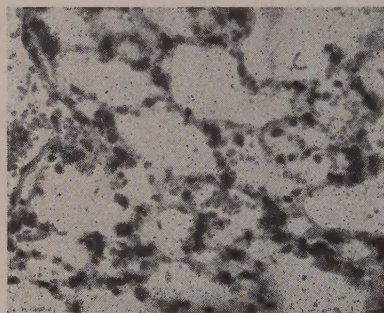


FIG. 3.

of the enzyme coincides with that of the zymogen granules, while the islets of Langerhans are entirely negative (Fig. 2). In the lung, the bronchial epithelium and the septal cells are stained selectively (Fig. 3). No lipase was demonstrated in the spleen, lymph nodes, brain and muscle.

The extent and intensity of the histochemical reaction is in good agreement with the results of chemical determinations of lipase activity in the same organs. Dog liver, rat

kidney and rat pancreas which show a very intense and extensive histochemical picture were found to liberate 1.6 to 2.6 mM of butyric acid in 2 hr per g of tissue from methyl-butyrate buffered at pH 7.6. Rat lung and dog kidney which show much less activity in the slide were found to liberate 0.45 to 0.84 mM of acid under the same conditions. Rat testicle and guinea pig kidney which show even less activity microscopically, liberated 0.15 to 0.35 mM of acid. Finally, brain, muscle and lymphatic tissue, negative histologically, showed only traces of lipase activity in the test tube (less than 0.05 mM of acid).

The reaction is completely prevented by treating the slide before incubation with Lugol's iodine solution for one minute, or with 5% phenol or with boiling water for 10 min.

When the effect of bile salts on the lipase picture of various tissues was studied, it was found that the addition of 0.2% Na taurocholate to the incubating mixture lead to a considerable intensification of the reaction in the pancreas, while in all other organs the enzyme was greatly inhibited. This is in good agreement with known differences in the bile salt activation of pancreatic and other lipases.⁹

Summary. Lipase can be demonstrated microtechnically in paraffin sections of acetone-fixed tissues. The slides are incubated with a water-soluble ester of palmitic or stearic acid in the presence of CaCl_2 . At the sites of lipase activity, the insoluble Ca palmitate or stearate precipitates can be visualized by transformation into the corresponding lead salts and subsequent blackening with H_2S .

⁹ Willstaetter, R., and Mennen, F., *Z. physiol. Chem.*, 1924, **138**, 216.

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the June, 1945 number of the PROCEEDINGS.

End